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(21) International Application Number: PCT/DK90/00225 (22) International Filing Date: 29 August 1990 (29.08.90) (30) Priority data: 4235/89                      29 August 1989 (29.08.89)      DK 4236/89                      29 August 1989 (29.08.89)      DK 4237/89                      29 August 1989 (29.08.89)      DK (71) Applicant (for all designated States except US): NOVO NORDISK A/S [DK/DK]; Novo Alle, DK-2880 Bagsværd (DK). (72) Inventors; and (75) Inventors/Applicants (for US only) : ESPER, Boel [DK/DK]; Lyngbakkevej 25, Søllerød, DK-2840 Holte (DK). LARS, Sottrup-Jensen [DK/DK]; Johannes Evaldsvej 75, DK-8230 Åbyhøj (DK).			(74) Common Representative: NOVO NORDISK A/S; Patent Department, Novo Alle, DK-2880 Bagsværd (DK). (81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent)*, DK (European patent), ES (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US. Published <i>With international search report.</i>
(54) Title: EXPRESSION OF ALPHA-MACROGLOBULINS			
(57) Abstract <p>α-Macroglobulins, especially human α<sub>2</sub>-macroglobulin, variants, fragments or derivatives thereof is produced by recombinant technology. The products are useful as additives to growth media, as proteinase inhibitors, as carrier in enzyme replacement therapy, and as DNA carrier in gene therapy.</p>			

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**Title:** Expression of alpha-macroglobulins

### **FIELD OF THE INVENTION**

The present invention relates to the expression of  $\alpha$ -macroglobulins, derivatives and variants thereof, and especially the expression of the human  $\alpha_2$ -macroglobulin ( $\alpha_2$ M) in an active form in mammalian cells, and the expression of genetically engineered variants thereof. The use of such recombinant  $\alpha$ -macroglobulins, especially recombinant  $\alpha_2$ M( $r\alpha_2$ M) and variants is described with examples from the fields of medicine for therapeutic purposes, and the development of novel defined growth media for propagation of mammalian cells in culture.

### **BACKGROUND OF THE INVENTION.**

#### **BIOCHEMISTRY OF $\alpha_2$ -MACROGLOBULIN ( $\alpha_2$ M).**

15           The proteinase binding glycoprotein  $\alpha_2$ M, which is synthesized in the liver, constitute together with the complement proteins C3, C4 and C5 a separate class of structurally and functionally related large plasma proteins. For a recent review see (Sottrup-Jensen, L. (1987) in: The Plasma Proteins (Putnam, F.W., ed.) 2nd Ed., 5: 191-291, Academic Press, Orlando, 20 FL).

Apart from C5 these proteins contain an internal B-cysteinyll- $\gamma$ -glutamyl thiol ester, which enables the proteolytically activated forms of  $\alpha_2$ M, C3, and C4 to participate in characteristic covalent binding reactions (Sottrup-Jensen, L., et al., (1980) FEBS Lett. 121: 275-280; Salvesen, G.S. 25 and Barrett, A.J., (1981) Biochem. J. 187: 695-701). The thiol ester structure, which in the active proteins can be slowly cleaved by a number of small nitrogen nucleophiles, constitutes a unique type of postsynthetic modification of proteins, and plays a prominent role in the biological properties of  $\alpha_2$ M. The presence of the active thiol esters in  $\alpha_2$ M is revealed 30 by a characteristic pattern of heat fragmentation (Harpel, P.C., et al., (1979) J. Biol. Chem. 254: 8869-8878).

Traditionally,  $\alpha_2$ M has been studied within the context of plasma proteinase inhibitors, although by several criteria it is unique. Whereas most plasma proteinase inhibitors are monomeric proteins of roughly similar 35 size, containing approximately 430-500 residues,  $\alpha_2$ M is a tetramer whose 180-kD subunits contain 1451 residues (Sottrup-Jensen et al., (1984) J. Biol. Chem. 259: 8318-8327).

Furthermore, in contrast to most other proteinase inhibitors, which form 1:1 complexes with serine proteinases engaging the active site

of the proteinase and the reactive site of the inhibitor,  $\alpha_2$ M forms complexes with a broad spectrum of proteinases differing in their substrate specificity and catalytic mechanism e.g.: trypsin, leucocyte elastase, chymotrypsin, pancreatic elastase, cathepsin G, plasmin, plasma kallikrein and thrombin.

5 The second-order rate constant for association between these proteinases and  $\alpha_2$ M varies by several orders of magnitude. Both 1:1 and 2:1 proteinase- $\alpha_2$ M complexes can be formed, and the disulfide-bridged dimer (360 kD) appears to be the functional unit of  $\alpha_2$ M (Sottrup-Jensen, L. (1987) in: The Plasma Proteins (Putnam, F.W., ed.) 2nd Ed., 5: 191-291, Academic Press, 10 Orlando, FL). Contrary to "classical" proteinase inhibitor complexes the  $\alpha_2$ M bound proteinase is still active, especially toward small synthetic substrates (Sottrup-Jensen, L. (1987) in: "The Plasma Proteins" (Putnam, F.W., ed.) 2nd Ed., 5: 191-291, Academic Press, Orlando, FL).

The mechanism of proteinase binding by  $\alpha_2$ M has been described by 15 the "trap" (Barrett, A.J. and Starkey, P.M. (1973) Biochem. J. 133: 709-724), where proteolytic cleavage of a particularly exposed peptide stretch near the middle of the 180-kD subunit (the "bait" region) results in a conformational change of the  $\alpha_2$ M tetramer, thereby entrapping the proteinase. The nature of the essentially irreversible proteinase complex formation 20 with  $\alpha_2$ M has long remained elusive. However, recent investigations show that a major fraction (typically > 80-90 % of the trapped proteinase is also covalently bound through epsilon-lysyl(proteinase)- $\gamma$ -glutamyl( $\alpha_2$ M) bonds (Sottrup-Jensen, L. et al., (1981) FEBS Lett. 128: 127-132; Sand, O. et al., (1985) J. Biol. Chem. 260: 15723-15735; Pochon, F. et al., (1987) FEBS Lett. 217: 25 101-105).

#### PHYSIOLOGICAL ASPECTS OF PROTEINASE- $\alpha_2$ M INTERACTIONS.

Since the  $\alpha_2$ M-proteinase complexes are rapidly cleared from the circulation (Ohlsson, K. (1971) Acta Physiol. Scand. 81: 269-272; Imber, 30 M.J. and Pizzo, S.V. (1981) J. Biol. Chem. 256: 8134-8139.) a general role as a "clearing vehicle" for plasma proteinases has been envisaged.

The main physiological targets may include proteinases of the coagulation and fibrinolysis systems and plasma kallikrein, and perhaps also proteinases like leucocyte elastase, cathepsin G and collagenases and other 35 proteinases released during cellular turnover (Sottrup-Jensen, L. and Birkedal-Hansen, H. (1989) J. Biol. Chem. 264: 393-401).

Although  $\alpha_2$ M may be largely confined to the vasculature in healthy uninflamed tissues, the inhibitor and its proteinase complexes are found at near plasma levels in inflammatory exudates of rheumatoid joints and gingival

crevicular fluids (Tollefsen, T. and Saltved, E. (1980) J. Periodont. Res. 15: 96-106; Borth, W., et al., (1983) Ann. N. Y. Acad. Sci. 421: 377-381).

While plasma  $\alpha_2$ M appear to be synthesized in the liver (Schreiber, G. (1987) in: "The Plasma Proteins" (Putnam, F.W., ed) 2nd Ed., 5: 294-363, Academic Press, Orlando, FL.) other sites of synthesis exist. Several cell strains in culture have been shown to produce  $\alpha_2$ M including fibroblasts (Mosher, D.F., et al., (1977) J. Clin. Invest. 60: 1036-1045) and monocytes-/macrophages (Hovi, T., et al., (1977) J. Exp. Med. 145: 1580-1589).

Whereas hepatocytes and Kupffer cells of the liver are most important for clearance of  $\alpha_2$ M-proteinase complexes in plasma (Davidsen, O., et al., (1985) Biochim. Biophys. Acta 846: 85-92), fibroblasts (Van Leuven, F., et al., (1979) J. Biol. Chem. 254: 5155-5160; Mosher, D.F. and Vaheri, A. (1980) Biochim. Biophys. Acta 627: 113-122) and macrophages (Debanne, M.T., et al., (1975) Biochim. Biophys. Acta 411: 295-304; Kaplan, J. and Nielsen, M.L. (1979) J. Biol. Chem. 254: 7323-7328) also possess receptors for  $\alpha_2$ M-proteinase complexes.

These observations suggest that there may be a considerable extravascular turnover of  $\alpha_2$ M perhaps primarily carrying proteinases functioning in the cellular micro environment (Sottrup-Jensen, L. and Birkedal-Hansen, H. (1989) J. Biol. Chem. 264: 393-401).

#### SUMMARY OF THE INVENTION

Briefly stated, the present invention discloses a method for the production of recombinant  $\alpha$ -macroglobulins, and especially human  $\alpha_2$ M, and variants thereof in an active form.

Within a preferred embodiment, the cultured host cell is an eukaryotic cell such as a mammalian cell or cells derived from organisms such as insects, plants, yeast or other fungi, such as Aspergillus.

The invention further relates to DNA sequences comprising a gene encoding for the expression of human  $\alpha_2$ M and variants thereof, vectors comprising such DNA sequences, and suitable hosts transformed with such vectors.

Yet another aspect of the invention is the use of recombinant  $\alpha_2$ M and variants thereof as a protein carrier in enzyme replacement therapy (ERT).

Yet another aspect of the invention is the use of recombinant  $\alpha_2$ M and variants thereof as a DNA carrier in gene therapy.

Further aspects of the invention relates to the use of recombinant  $\alpha$ -macroglobulins, especially human  $\alpha_2$ M, and variants thereof as

constituents of growth media, either as an additive or co-expressed with a desired gene product.

### DEFINITIONS

5 Prior to setting forth the invention it may be helpful for an understanding thereof to set forth definitions of certain terms to be used hereafter.

**Complementary DNA or cDNA:** A DNA molecule or sequence which have been  
10 enzymatically synthesized from sequences present in a mRNA template.

**DNA Construct:** A DNA molecule, or a clone of such a molecule, either single-  
or double-stranded, which may be isolated in partial form from a naturally  
occurring gene or which has been modified to contain segments of DNA which  
15 are combined and juxtaposed in a manner which would not otherwise exist in  
nature.

**Plasmid or Vector:** A DNA construct containing genetic information which may  
provide for its replication when inserted into a host cell. A plasmid  
20 generally contains at least one gene sequence to be expressed in the host  
cell, as well as sequences encoding functions which facilitate such gene  
expression, including promoters and transcription initiation sites. It may  
be a linear or closed circular molecule.

25 **Joined:** DNA sequences are said to be joined when the 5' and 3' ends of one  
sequence are attached by phosphodiester bonds to the 3' and 5' ends,  
respectively, of an adjacent sequence. Joining may be achieved by such  
methods as ligation of blunt or cohesive termini, by synthesis of joined  
sequences through cDNA cloning, or by removal of intervening sequences  
30 through a process of directed mutagenesis.

**Variant:** A peptide related to the original peptide, but wherein the amino  
acid sequence has been altered through mutation of the gene encoding the  
original peptide.

**ABBREVIATIONS****AMINO ACIDS**

A	=	Ala	=	Alanine
V	=	Val	=	Valine
5 L	=	Leu	=	Leucine
I	=	Ile	=	Isoleucine
P	=	Pro	=	Proline
F	=	Phe	=	Phenylalanine
W	=	Trp	=	Tryptophan
10 M	=	Met	=	Methionine
G	=	Gly	=	Glycine
S	=	Ser	=	Serine
T	=	Thr	=	Threonine
C	=	Cys	=	Cysteine
15 Y	=	Tyr	=	Tyrosine
N	=	Asn	=	Asparagine
Q	=	Gln	=	Glutamine
D	=	Asp	=	Aspartic Acid
E	=	Glu	=	Glutamic Acid
20 K	=	Lys	=	Lysine
R	=	Arg	=	Arginine
H	=	His	=	Histidine

**NUCLEIC ACID BASES**

25 A	=	Adenine
G	=	Guanine
C	=	Cytosine
T	=	Thymine(only in DNA)
U	=	Uracil (only in RNA)

30

**BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1a illustrates the construction of plasmid p1136.

Figure 1b illustrates the construction of plasmid p1167.

35 Figure 2 illustrates the structure of plasmid p1167.

Figure 3 illustrates a gel electrophoresis (10 - 20 % SDS-PAGE) of the thermal fragmentation products generated from  $\alpha_2$ M and  $\alpha_2$ M.

Figure 4 illustrates a gel electrophoresis of the thermal fragmentation products generated from methylamine treated  $\alpha_2$ M and  $\alpha_2$ M.

Figure 5 illustrates a gel electrophoresis (SDS-PAGE) of the reaction products generated from trypsin treatment of  $\alpha_2M$  and  $\alpha_2M$ .

Figure 6 illustrates a gel electrophoresis of the reaction products generated from trypsin treatment of methylamine-treated  $\alpha_2M$  and  $\alpha_2M$ .

5 Figure 7 illustrates a "rate gel" electrophoresis of unreacted native -and trypsin treated  $\alpha_2M$  and  $\alpha_2M$ .

Figure 8 illustrates a "rate gel" electrophoresis of unreacted native -and methylamine treated  $\alpha_2M$  and  $\alpha_2M$ .

Figure 9 illustrates the chromatograms of  $\alpha_2M$  and  $\alpha_2M$  on a 10 Superose 6 column.

Figure 10 illustrates the gel electrophoresis (10 - 20 % reducing SDS-PAGE) of the reaction products from chymotrypsin treated human  $\alpha_2M$ , human PZP and  $\alpha_2M$ -PZP.

15 Figure 11 illustrates the gel electrophoresis (10 - 20 % reducing SDS-PAGE) of the reaction products from elastase treated human  $\alpha_2M$ , human PZP and  $\alpha_2M$ -PZP.

Figure 12 illustrates the gel electrophoresis (10 - 20 % reducing SDS-PAGE) of the reaction products from trypsin treated human  $\alpha_2M$ , human PZP and  $\alpha_2M$ -PZP.

20 Figure 13 illustrates the gel electrophoresis (10 - 20 % reducing SDS-PAGE) of the reaction products from Staphylococcus aureus Glu-specific protease treated human  $\alpha_2M$ , human PZP and  $\alpha_2M$ -PZP.

## 25 DETAILED DESCRIPTION OF THE INVENTION

According to the invention there is provided a process for the production of  $\alpha$ -macroglobulins, especially human  $\alpha_2$ -macroglobulin, or fragments or derivatives, including variants thereof, wherein a functionally operative expression vector comprising a gene encoding for the expression of 30 a  $\alpha$ -macroglobulin, especially human  $\alpha_2$ -macroglobulin, or fragments or derivatives thereof, including variants, or alleles of such a gene, is introduced into a suitable host capable of expressing said gene, said host is cultured in a suitable nutrient medium containing sources of assimilable carbon and nitrogen and other essential nutrients, and the expressed  $\alpha$ - 35 macroglobulin, especially human  $\alpha_2$ -macroglobulin, or fragments or derivatives thereof is recovered.

Many proteins synthesized particularly in mammalian cells undergo post-translational modification (processing) of one kind or the other.



Depending on the final destination and on the specific function of a newly synthesized protein, it may go through a number of processing steps leading to covalent modifications such as e.g.: glycosylation,  $\gamma$ -carboxylation,  $\beta$ -hydroxylation, sulphatation, amidation, thiol ester formation, phosphorylation, proteolytic cleavage at precursor processing sites, fatty acylation (Rosner, M.R. (1986). in: "Mammalian Cell Technology", (Thilly, W.G. ed), Butterworth Publishers, Stoneham, MA.: 63-89).

Proteins of various sizes and with a variety of different post-translational modifications have been successfully expressed in transformed heterologous mammalian host cells using recombinant DNA technology. A few examples: Human coagulation factors VIIa and IX have been expressed in transformed BHK (Syrian Baby Hamster Kidney) cells with correct post-translational modifications such as  $\gamma$ -carboxylation and glycosylation (Thim, L. et al., (1988) Biochemistry 27: 7785-7793; Busby, S. et al., (1985) Nature 316: 271-273). Human Platelet-derived Growth Factor AB heterodimer has been expressed in transformed CHO (Chinese Hamster Ovary) cells with correct processing of the A and B chain precursors and correct assembly of the AB heterodimer. Human coagulation factor VIII has been expressed in transformed CHO cells with correct processing of the precursor leading to a two chain molecule that can be activated by thrombin and factor Xa (Kaufman, R.J. et al., (1988) J. Biol. Chem. 263: 6352-6362; Pittman, D.D. and Kaufman, R.J. (1988) Proc. Natl. Acad. Sci. USA 85: 2429-2433).

So far, there have been no reports on the heterologous expression of proteins in which the formation of an active thiol ester is a prominent post-translational modification.

The biosynthesis of the internal thiol ester in the third component (C3) of complement from rabbit has been investigated (Iijima, M. et al., (1984) J. Biochem. 96: 1539-1546). Rabbit liver mRNA was translated in vitro in a rabbit reticulocyte lysate system, and the synthesized C3 specific products did not incorporate radio labelled methylamine. On the other hand radio labelled iodoacetamide reacted with the synthesized C3 specific products; these results indicated the presence in the primary C3 specific translation product of a free thiol group instead of a reactive thiol ester. If a liver homogenate supernatant (S-13) including cytosol and microsomes was included, the C3 specific product could now incorporate methylamine. By increasing the concentration of the S-13 component(s), the incorporation of methylamine in C3 specific products was increased, and at the same time incorporation of iodoacetamide decreased. If the S-13 fraction was treated at 65°C for 5 min, the activity was completely lost.

The results from this investigation strongly suggest an involvement of a transglutaminase-like or other type of enzyme in the posttranslational formation of an active thiol ester in rabbit C3. There are no similar investigations addressing the formation of the thiol ester in other  $\alpha$ -macroglobulins, e.g.  $\alpha_2$ M, but from analogy and homology considerations, it is expected that a similar mechanism is responsible for the formation of thiol esters in other  $\alpha$ -macroglobulins synthesized in the mammalian liver.

Through this investigation a number of developments were done which also are deemed to be encompassed of the present invention. These include DNA sequences comprising a gene encoding for the expression of  $\alpha$ -macroglobulins, especially human  $\alpha_2$ -macroglobulin, or fragments or derivatives and variants thereof as exemplified in SEQ ID NO:1 and SEQ ID NO:3.

Another aspect of the invention relates to functionally operative expression vectors comprising a gene encoding for the expression of at least one  $\alpha$ -macroglobulin, especially human  $\alpha_2$ -macroglobulin or fragments or derivatives and variants thereof, or alleles of such a gene.

Such vectors preferably further comprise regulatory elements necessary for the stable maintenance of said vector in mammalian cells.

Also, such vectors may further include sequences providing for the processing and secretion of the expressed product.

In relation to the use of recombinant  $\alpha$ -macroglobulins, and especially  $\alpha_2$ M, in growth media it may be co-expressed with another desired gene product, and consequently the vectors of the invention may further comprise one or more other genes encoding for a desired gene product.

The invention further relates to transformed hosts comprising a functionally operative expression vector according to the invention comprising a gene encoding for the expression of human  $\alpha_2$ -macroglobulin or fragments or derivatives and variants thereof, or alleles of such a gene.

The host may be selected from the group comprising a bacterial strain, a fungal strain, a mammalian cell line, or a mammal, especially a fungus, such as belonging to the genus Aspergillus, or a yeast strain, preferably belonging to the genus Saccharomyces.

Another preferred type of host is a mammalian cell line, preferably a Syrian Baby Hamster Kidney (BHK) cell line, and especially the one which is available from ATCC under No. CRL 1632.

The invention further relates to the recombinant human  $\alpha_2$ -macroglobulin or a variant thereof in an active form having the amino acid sequence of SEQ ID NO:2, or SEQ ID NO:4.

#### 5 APPLICATIONS OF $\alpha$ -MACROGLOBULINS, ESPECIALLY $\alpha_2$ M.

The present invention discloses applications of  $\alpha$ -macroglobulins, and especially  $\alpha_2$ M. These should be regarded not as limitations but as a few examples among many for the use of recombinant derived  $\alpha$ -macroglobulins.

#### 10 $\alpha$ -MACROGLOBULINS AS CONSTITUENTS OF DEFINED GROWTH MEDIA.

Degradation of specific heterologous products produced in either transformed or non-transformed mammalian cells is a potential problem in the production of recombinant products. This is due to the fact that many host cells secrete one or more different proteinases.

15 When a production cell line is grown in the presence of e.g. 10 % fetal calf serum, such proteolytic degradation of secreted recombinant or native protein products is a minor problem due to a buffering effect of the added serum proteins.

However, the use of fetal calf serum in the large scale growth  
20 (fermentation) of mammalian production cell lines is not a desirable situation for a number of reasons. First of all fetal calf serum is a very costly constituent of complex growth media; second, the demand for fetal calf serum from a growing biopharmaceutical industry might not be easily fulfilled in the future, and third, the use of fetal calf serum constitutes  
25 a potential quality control problem in the production of pharmaceuticals intended for use in humans.

To circumvent these problems, efforts can be expected in the field of development of defined growth media for use with mammalian cells.

Addition of various proteinase inhibitors to such new defined  
30 growth media will be required to ensure the integrity of the secreted products. Alternatively, the producer cell line might, through genetic engineering, be endowed with the capacity to produce and secrete proteinase inhibitors along with the desired product(s).

$\alpha$ -Macroglobulins, and especially Human  $\alpha_2$ M, are proteinase  
35 inhibitors of broad specificity, and they are therefore according to the invention used as constituents of defined growth media for mammalian cells, either as a medium additive or as a product co-produced with the desired product.

The target sites for a number of different proteinases, e.g. bovine trypsin, Streptomyces griseus trypsin, papain, porcine elastase, bovine chymosin, bovine chymotrypsin, Staphylococcus aureus strain V8 proteinase, human plasmin, bovine thrombin, thermolysin, subtilisin Novo and 5 Streptomyces griseus proteinase B have been mapped in the bait region of human  $\alpha_2$ M (Mortensen, S.B., et al., (1981) FEBS Lett. 135: 295-300) and other  $\alpha$ -macroglobulins (Sottrup-Jensen, L., Sand, O., Kristensen, L. and Fey, G.H. J. Biol. Chem. 264, 15781-15789, 1989). It is evident that  $\alpha_2$ M and the other  $\alpha$ -macroglobulins as proteinase inhibitors have broad specificities.

10 In those situations, where the proteinase inhibitory spectrum of a  $\alpha$ -macroglobulin, such as  $\alpha_2$ M, is not sufficient for the prevention of product degradation, it is possible through site specific mutation, protein engineering, etc. to change the proteinase inhibitor specificity of the  $\alpha$ -macroglobulin, such as  $\alpha_2$ M. Incorporation of desirable specific proteinase 15 target sites in the bait region of recombinant  $\alpha_2$ M will change the inhibitor specificity of the mutated  $\alpha_2$ M. Furthermore it is possible through genetic engineering to construct novel specific or general proteinase target sites in the bait region of a  $\alpha$ -macroglobulin in order to enhance its versatility as a proteinase inhibitor of specific or broad inhibitory spectrum. 20 Furthermore it is possible to remove specific target sites in an  $\alpha$ -macroglobulin in order to avoid degradation of the variant in question by certain proteases in the circulation that will already be inhibited through the action of naturally present proteinase inhibitors.

The production of recombinant products in fungi, such as species 25 and strains of e.g. Aspergillus and Saccharomyces also meets with potential problems of product degradation. In some cases it is possible to isolate proteinase negative mutants of desirable production strains. This might not always be the case, and co-expression of  $\alpha$ -macroglobulins, such as  $\alpha_2$ M or  $\alpha_2$ M-mutants together with a desirable product may inhibit proteolysis of the 30 product in question.

#### $\alpha$ -MACROGLOBULIN MUTANTS AS SPECIFIC PROTEINASE INHIBITORS.

The amino acid sequence of the bait region of  $\alpha$ -macroglobulins defines the specificity of the  $\alpha$ -macroglobulin towards different proteina- 35 ses. A comparison of cleavage patterns for different proteinases and bait region sequences in five mammalian  $\alpha$ -macroglobulins has recently been published (Sottrup-Jensen, L., Sand, O., Kristensen, L. and Fey, G.H. The  $\alpha$ -macroglobulin bait region. Sequence diversity and localization of cleavage sites for proteinases in five mammalian  $\alpha$ -macroglobulins. J. Biol. Chem. 264,

15781-15789, 1989). It has previously been clearly demonstrated that the bait region in each species of  $\alpha$ -macroglobulin is the major determinant of proteinase inhibitor specificity. The present invention demonstrates the possibility of modulating the inhibitor specificity of human  $\alpha_2$ M by alterations of proteinase target sites in the bait region.

In the present invention it is demonstrated that the bait region of human  $\alpha_2$ M (residues 690 to 730 in SEQ ID NO:2) can be mutated at will to obtain a new proteinase inhibitor profile of this macroglobulin. The example presented in the present invention describes the construction of a hybrid macroglobulin. In this hybrid the bait region from human pregnancy zone protein (PZP) was introduced into human  $\alpha_2$ M, from which the native bait region had been removed. The hybrid molecule, which was constructed by the use of recombinant DNA technology, revealed a proteinase inhibitor profile similar to the inhibitor profile of PZP.

15 The invention thus demonstrates the possibility to design and produce proteinase inhibitors with altered and new inhibitor specificities at will.

This finding is important for the design of new proteinase inhibitors. Due to the low antigenicity the bait region in macroglobulins (Van Leuven, F., Marynen, P., Cassiman, J.-J. and Van den Berghe, H. Mapping of structure-function relationships in proteins with a panel of monoclonal antibodies. A study on human alpha-2-macroglobulin. J. Immunol. Methods 111, 39-49, 1988, and Delain, E., Barray, M., Tapon-Brethaudiere, J., Pochon, F., Marynen, P., Cassiman, J.-J., Van den Berghe, H. and Van Leuven, F. The Molecular Organization of Human alpha2-Macroglobulin. An Immunoelectron microscopic study with monoclonal antibodies. J. Biol. Chem. 263, 2981-2989, 1988) it is now possible, by the use of the technology described in the present invention, to design non-immunogenic new proteinase inhibitors that can be used e.g. in the treatment of any disease, where aggressive proteinases constitute a threat to the health of man.

In the present specification the production of  $\alpha_2$ M variants is described by the construction of a hybrid macroglobulin. It is clear to the skilled person in the art that changes also could be obtained through other genetic engineering methods, such as described in International Publication No. WO 89/06279 (NOVO INDUSTRI A/S). Also it is clear that other  $\alpha$ -macroglobulins could be employed instead of the human  $\alpha_2$ M, such as those mentioned in Sottrup-Jensen, L. et al. (1989), supra.

$\alpha_2$ M AS A PROTEIN CARRIER IN ENZYME REPLACEMENT THERAPY.

A different application of  $\alpha_2$ M is its use as a carrier of macromolecules such as proteins and nucleic acids. When  $\alpha_2$ M reacts with and forms a complex with a proteinase in solution,  $\alpha_2$ M may bind other proteins (also 5 non-proteinase proteins) present in that solution (Salvesen, G.S. et al., (1981) Biochem. J. 195: 453-461). In the case of Fabry's disease, which is an X-chromosome linked disorder of glycosphingolipid metabolism, it has recently been demonstrated that  $\alpha_2$ M can function as a carrier in an in vitro model of enzyme replacement therapy (ERT) (Osada, T., et al., (1987) Biochem. 10 Biophys. Res. Commu. 142: 100-106).  $\alpha_2$ M was conjugated to coffee bean  $\alpha$ -galactosidase through the action of trypsin, and the formed complex was internalized through  $\alpha_2$ M-receptor specific (Van Leuven, F., et al., (1981) J. Biol. Chem. 256: 9016-9022) endocytosis and delivered to the lysosomes, which is the target organelle for  $\alpha_2$ M-receptor mediated internalization of  $\alpha_2$ M-15 proteinase complexes (Willingham, M.C. and Pastan, I., (1980) Cell 21: 67-77).

Such a scheme in ERT provides a method of internalization to the lysosome of the enzyme in question and at the same time it might alleviate potential antigenicity problems arising from the use of heterologous enzymes 20 in therapy. One limitation in this type of ERT (Osada, T., et al., (1987) Biochem. Biophys. Res. Commu. 142: 100-106) would be the types of potential target cells that could be treated by this protocol. Obviously, they would have to express the  $\alpha_2$ M-receptor. In a future development of the system, the possibility might exist to redesign the cell specificity of  $\alpha_2$ M internaliza- 25 tion by exchanging the receptor binding domain of  $\alpha_2$ M with other receptor ligands. Hereby  $\alpha_2$ M-mutants could be designed to enter any cell type known to express a specific internalizable receptor.

This type of development would of course require a system for the production of recombinant derived  $\alpha_2$ M. The use of native human  $\alpha_2$ M as a 30 carrier in ERT (as described above) is undesirable due to the now well known risks of the employment of blood derived products in the treatment of human disease.

The production of recombinant  $\alpha_2$ M in accordance with the present invention alleviates this problem by providing for large scale production 35 of  $\alpha_2$ M.

 $\alpha_2$ M AS A DNA CARRIER IN GENE THERAPY.

Advances in gene transfer into mammalian cells have opened for the possibility of the treatment of a number of genetic disorders through

gene therapy. A major problem in gene therapy will be the specific targeting of genes into the appropriate cells within the body. (Williamson, B., (1982) *Nature* 298: 416-418; Anderson, W.F., (1984) *Science* 226: 401-409; Parkman, R., (1986) *Science* 232: 1373-1378).

5 It was recently described that a constructed foreign gene containing the chloramphenicol acetyltransferase (CAT) on a bacterial plasmid could be targeted to the liver of rats by specific receptor directed internalization (Wu, G.Y. and Wu, C.H. (1988) *J. Biol. Chem.* 263: 14621-14624). The DNA carrier consisted of a galactose-terminal (asialo)glyco-  
10 protein and asialoorosomucoid covalently linked to poly-L-lysine. The polycation poly-L-lysine can bind DNA in a strong non-covalent and nondamaging interaction. It was demonstrated that complex bound DNA was internalized by cell-surface asialoglycoprotein receptors that are unique to hepatocytes. The complex was injected intravenously, and upon analysis only the liver  
15 expressed the CAT activity.

In the present invention the use of  $\alpha_2M$  as a carrier of DNA in gene therapy is suggested. Reaction of  $\alpha_2M$  with a proteinase such as trypsin or with methylamine in the presence of covalently closed circular plasmid DNA is likely to result in partial or total entrapment of DNA within the  
20 complexing  $\alpha_2M$  molecule. After intravenous injection of such complexes with exposed receptor binding domains, the complex will be rapidly cleared from the blood and internalized in specific target cells, such as hepatocytes and Kupffer cells. Through protein engineering on the receptor binding domain of  $\alpha_2M$  it will be possible to design a DNA carrier specific for other cell  
25 types. The advantage in this system as compared to the above described system using the asialoglycoprotein receptor is, that it will not be necessary to identify different DNA carrier systems for each new cell type.

### 30 EXAMPLES

#### Materials and methods:

##### Microorganisms and cell lines

E. coli K12 (MC1061) is available from e.g. Stratagene Inc.,  
35 11099 North Torrey Pines Rd., La Jolla, California 92037.

HepG2 (Human hepatoblastoma cell line) is freely available from American Type Culture Collection, under No. HB 8065.

BHK (Syrian Hamster Kidney cell line, thymidine kinase mutant line tk<sup>-</sup>13, (Waechter and Baserga (1982) *Proc. Natl. Acad. Sci. USA* 79:

1106-1110); is freely available from American Type Culture Collection, under No. CRL 1632.

### Plasmids and vectors

5

Plasmids pCDVI-PL and pSP62-K2 are available from Dr. Tasuku Honjo, Faculty of Medicine, Kyoto University, Kyoto 606, Japan. pSP62-K2 was derived from the plasmid pSP62-PL (available from New England Nuclear/Du Pont (U.K.) Ltd., Wedgwood Way, Stevenage, Hertfordshire, SG14QN) as described (Noma et al., (1986) Nature, 319: 640-646). pCDVI-PL was derived from pcDV1 (Okayama, H. and Berg, P. (1983) Molec. cell. Biol. 3: 280-289) as described (Noma et al., (1986) Nature, 319: 640-646).

M13mpl8 is available from Pharmacia LKB Biotechnology (catalog # 27-1552-01) (Norranders, J., Kempe, T. and Messing, J. Gene 26: 101-106, 15 1983).

M13mpl9 is available from e.g. International Biotechnologies, Inc., P.O. Box 9558, 275 Winchester Avenue, New Haven, Connecticut 06535, USA.

pDHFR-I is available from Dr. K.L. Berkner, ZymoGenetics Inc., 20 4225 Roosevelt Way NE, Seattle, Washington 98105. (The construction of this plasmid is given in detail in: Berkner, K.L. and Sharp, P.A. (1984) Nucleic Acids Res. 12: 1925-1941). The molecular cloning of the DHFR cDNA present in this plasmid, and its sub-cloning in mammalian expression vectors under the control of adenovirus derived promoters has previously been described 25 in detail (Chang, A.C.Y., et al., Nature 275: 617-624 and Kaufman, R.J. and Sharp, P.A. (1982) Mol. Cell. Biol. 2: 1304-1319). The backbone plasmid in pDHFR-I is pBR322 (Sutcliffe, J.G. (1979) Cold Spring Harbor Symp. Quant. Biol. 43: 77-90; Sutcliffe, J.G. (1978) Nucleic. Acids Res. 5: 2721-2728).

pUC13 is described in: Vieira, J. and Messing, J.: 1982, Gene 19: 30 259-268 and available from Pharmacia LKB Biotechnology (catalog # 27-4954-01).

pUC19 is described in: Yanisch-Perron, C. and Messing, J., 1985, Gene 33:103-119 and available from Pharmacia LKB Biotechnology (catalog # 27-4951-01).

35



**Growth media****LB-broth:****Mix**

227 g Bacto Tryptone, Difco 0123-01

113.5 g Yeast extract, Difco 0127-01, and

5 227 g NaCl in a sealable plastic container.

Add 12.5 g mix to 500 ml water in a 1000 ml bottle, shake well and sterilize in an autoclave.

Dulbeccos Modified Eagle Medium is available from e.g. Gibco Ltd. 10 P.O. Box 35, Trident House, Renfrew Road, Paisley PA34EF, Renfrewshire, Scotland. Cat.# 042-250 1M (10 \* concentrate).

**Antibodies**

15 Anti- $\alpha_2$ M A033 and peroxidase conjugated anti- $\alpha_2$ M PE326 were from DAKOPATTS A/S, Copenhagen, Denmark.

**EXAMPLE 1.****CLONING AND SEQUENCE DETERMINATION OF HUMAN  $\alpha_2$ M**

20

**Preparation of messenger RNA from the human cell line HepG2.**

The human hepatoblastoma cell line HepG2 (American Type Culture Collection No. HB 8065, freely available) was used as a source for mRNA preparation. HepG2 cells were grown to a total cell number of  $15 \times 10^7$  in 25 Dulbecco's Modified Eagle medium containing 10% fetal calf serum and antibiotics.

Total RNA was isolated by the guanidinium thiocyanate method (Chirgwin et al., (1979) Biochemistry 18: 5293-5299) and purified by CsCl gradient centrifugation. A total of 3000  $\mu$ g RNA was obtained. mRNA was 30 isolated by use of an oligo(dT)-cellulose column (Aviv & Leder (1972) Proc. Natl. Acad. Sci. USA 69: 1408-1412). 60  $\mu$ g of mRNA was obtained after one cycle of affinity chromatography. After ethanol precipitation, this preparation of mRNA was resuspended in 10 mM Tris-HCl pH 7.5, 0.1 mM EDTA- $\text{Na}_2$  at a final concentration of 1  $\mu$ g/ $\mu$ l and stored at  $-80^\circ\text{C}$  for subsequent 35 use in the construction of a cDNA library.

**Construction of a cDNA library from HepG2 mRNA.**

A cDNA library was constructed in the pCDVI-PL/pSP62-K2 vectors (Noma et al., (1986) Nature, 319: 640-646. Available from Dr. Tasuku Honjo,

Faculty of Medicine, Kyoto University, Kyoto 606, Japan) by use of the methods described by Okayama & Berg (Mol. Cell. Biol. 2: 161-170 (1982); Mol. Cell. Biol. 3: 280-289 (1983)).

E. coli K12 (MC1061) (Casadaban & Cohen (1980) J. Mol. Biol. 5 138: 179-207) was used for transformation. MC1061 were grown in L-broth at 37°C to OD<sub>660</sub>=0.5. Twenty ml were centrifuged, and the pellet was resuspended in 7 ml of ice-cold sterile 0.1 M CaCl<sub>2</sub>, incubated on ice for 30 minutes, centrifuged briefly, and finally kept in the cold room overnight.

Ninety-five µl suspension of transformation-competent E. coli 10 MC1061 were added per 10 µl of cDNA preparation. The mixture was incubated on ice for 30 minutes, heat-shocked at 43.5°C for 45 seconds, and finally, after addition of L-broth, incubated at 37°C for 30 minutes.

After resuspension, the cells were plated onto L-broth plates containing ampicillin (50 µg/ml) and grown for 8 hrs at 37°C. A total of 2.9 15 \* 10<sup>5</sup> individual colonies could be obtained from this library.

#### Screening of the HepG2 library for cDNA clones encoding human α<sub>2</sub>M.

5 \* 10<sup>4</sup> individual colonies were screened by standard colony hybridization technique using nitrocellulose filters (Maniatis et al., (1982) 20 Molecular Cloning - A Laboratory Manual, Cold Spring Harbor, New York).

A 20-mer oligonucleotide mixture

5' CC(T/C)TTCAT(G/A)TC(T/C)TC(T/C)TG(T/C)TT 3'

where the notation (X/Y) means that either of the nucleic acids X or Y may be used, complementary to the human α<sub>2</sub>M mRNA in the region encoding amino 25 acid residues Lys-Gln-Glu-Asp-Met-Lys-Gly (residues number 493 - 499 in Sottrup-Jensen et al., J. Biol. Chem. 259: 8318-8327 (1984) was synthesized (on a DNA synthesizer from Applied Biosystems, USA), labelled with <sup>32</sup>P (using T<sub>4</sub> polynucleotide kinase and γ-<sup>32</sup>P-ATP) to a specific activity of 3 \* 10<sup>8</sup> cpm/pmol oligonucleotide. The labelled oligonucleotides were purified by gel 30 chromatography and subsequently used in the screening of the cDNA library.

The hybridization solution contained 6 \* SSC, 5 \* Denhardt's solution, 0.05% SDS (Maniatis et al., (1982) Molecular Cloning - A Laboratory Manual, Cold Spring Harbor, New York) and 10<sup>6</sup> cpm/ml of labelled oligonucleotide mix.

35 Hybridization was performed for 3 hrs at 45°C. Then the filters were washed in 6 \* SSC, 0.05% SDS at 45°C for 3 \* 10 minutes. After autoradiography the filters were washed under the same conditions, but this time at 52°C. A colony that still showed hybridization at this temperature was isolated and the cDNA insert of the corresponding plasmid (designated pα<sub>2</sub>M)

from this isolate was sequenced (Tabor & Richardson (1987) Proc. Natl. Acad. Sci. USA 84: 4767-4771). The sequence of the cDNA and the derived encoded amino acid sequence are shown in the appended sequence listings, SEQ ID NO:1:, and SEQ ID NO:2:.

5

Characterization of p $\alpha_2$ M.

p $\alpha_2$ M had a cDNA insert of approximately 4.6 kb. Its sequence is given in Table I above.

10 The sequence in Table I demonstrates that the entire coding region of  $\alpha_2$ M including the signal peptide is found in the insert.

In addition to the coding region, the insert contains sequences derived from the 5'- and 3' untranslated regions of the  $\alpha_2$ M mRNA molecule.

The amino acid sequence of the human  $\alpha_2$ M as deduced from the cDNA  
15 in p $\alpha_2$ M is in total agreement with the published sequence (Sottrup-Jensen et al., (1984) J. Biol. Chem. 259: 8318-8327). Codon number 1000 (numbered from the initiating methionine codon in the signal peptide) was found to be ATC encoding an isoleucine and not GTC (encoding a valine) as found in an  $\alpha_2$ M cDNA synthesized from human liver mRNA (Kan et al., (1985) Proc. Natl. Acad. Sci.  
20 USA. 82: 2282-2286). In the  $\alpha_2$ M cDNA sequence from the HepG2 library we have further identified ten silent changes as compared to the sequence from the liver library, see the following Table I:

TABLE I

	Codon	Liver	HepG2
5	413 (Asn)	AAC	AAT
	495 (Phe)	TTT	TTC
10	750 (Gly)	GGG	GGT
	796 (Leu)	CTT	CTC
15	835 (Leu)	CTT	CTA
	1266 (Ala)	GCC	GCA
	1296 (Asn)	AAT	AAC
20	1326 (Thr)	ACC	ACA
	1442 (Leu)	CTC	CTG
25	1460 (Ile)	ATC	ATT

The position of the oligonucleotide mixture used as a hybridization probe in the colony screenings was from position 1574 to position 1594, and the position of the reactive thiol ester is from position 2939 to 2953 in SEQ ID NO:1.

#### EXAMPLE 2.

##### Construction of a mammalian expression vector for $\alpha_2$ M.

35  $\alpha_2$ M was digested (fig. 1a) with XbaI and EcoRI, and a 1.2 kb fragment containing the 5' part of the  $\alpha_2$ M cDNA together with the multiple cloning site of pSP62-K2 was isolated on an agarose gel and cloned in an XbaI/EcoRI digested M13mp19 vector to generate M13mp19A. To facilitate further subclonings of the  $\alpha_2$ M cDNA, a unique EcoRV site was introduced in 40 the 1.2 kb fragment 10 nucleotides 5' to the initiating ATG (methionine) codon through site directed mutagenesis (Kunkel et al., (1987) Methods Enzymol. 154: 367-382). In the same mutagenesis experiment, in which the mutagenic oligonucleotide NOR593:

5'(TTCTTCCCATGGTGGATATCGAAGGAGCTG)3'

45 was used, the 5 nucleotides 5' to the methionine codon was changed to CCACCATG; this mutation creates a new NcoI site spanning the ATG codon. A

correct mutant M13mp19B was identified through restriction enzyme digestion and DNA sequencing.

The mutated 5' end of  $\alpha_2M$  cDNA was isolated from M13mp19A replicative form through digestion with HindIII and EcoRI and agarose gel electrophoresis. The isolated DNA fragment was then joined to HindIII/EcoRI digested  $\alpha_2M$  through ligation to generate p1136. In this plasmid the  $\alpha_2M$  cDNA is reassembled in its total length, but now with a unique EcoRV site at the 5' end. p1136 was digested with EcoRV/DraI, and the  $\alpha_2M$  fragment was isolated on an agarose gel and cloned in a mammalian expression vector under control of the adenovirus 2 major late promoter (Ad 2 MLP).

The adenovirus-promoter based vector was constructed by K.L.Berkner (ZymoGenetics Inc., Seattle, WA.), and a detailed description of the functional elements in the mammalian expression vector is given in: Powell, J.S. et al., (1986) Proc. Natl. Acad. Sci. USA 83: 6465-6469 and in: Boel et al., (1987) FEBS Lett. 219: 181-188).

The expression vector used for expression of human  $\alpha_2M$  was generated from the mammalian expression vector pPP (Boel, E. et al., (1987) FEBS Lett. 219: 181-188), in which human pancreatic polypeptide cDNA was cloned under control of Ad 2 MLP.

20 pPP was digested (fig. 1b) with BamHI and the resulting staggered ends were repaired with DNA polymerase (Klenow fragment and the four deoxynucleotide triphosphates). The 4.5 kb EcoRV/DraI  $\alpha_2M$  cDNA fragment was joined to this vector through ligation, and correct recombinants were characterized through restriction enzyme analysis on isolated miniprep. 25 plasmids.

The  $\alpha_2M$ -mRNA transcribed from the resulting 8.76 kb plasmid (designated p1167 (fig. 2)) has the adenovirus 2 late tripartite leader (L1-3) at its 5' end together with an mRNA splice signal (SS). At the 3' end of the construct the transcript is terminated with the SV40 late termination - 30 and polyadenylation signal. 5' to the Ad 2 MLP the construct includes the SV40 enhancer (ENH) and the 0 to 1 (0 - 1) map units from adenovirus 5.

#### Expression of $\alpha_2M$ in mammalian cells.

For expression of human  $\alpha_2M$  in cultured BHK cells (Syrian Hamster 35 Kidney, thymidine kinase mutant line tk's13, (Waechter and Baserga (1982) Proc. Natl. Acad. Sci. USA 79: 1106-1110); American Type Culture Collection CRL 1632) the expression vector p1167 was co-transfected with pDHFR-I (Berkner, K.L. and Sharp, P.A. (1984) Nucleic Acids Res. 12: 1925-1941. Available from K.L.Berkner, ZymoGenetics Inc. Seattle) into subconfluent cells by the

calcium phosphate mediated transfection procedure (Graham and Van der Eb (1973) Virology 52: 456-467). In the transfection experiment the molar ratio between p1167 and pDHFR-I was 10:1. Cells were grown in Dulbeccos Modified Eagle Medium supplemented with 10% fetal calf serum (FCS).

5           Forty-eight hours after transfection, cells were trypsinized and diluted into medium containing 400 nM methotrexate (MTX). After 10 to 12 days, individual colonies were cloned out and expanded separately. The expanded cultures were propagated for 24 hours as described above, and producer clones were identified using an enzyme linked immunosorbent assays  
10 (ELISA) (Munck Petersen C., et al., (1985) Scand. J. Clin. Lab. Invest. 45: 735-740) against human  $\alpha_2$ M secreted to the growth medium.

Description of the  $\alpha_2$ M ELISA assay.

The materials used in the ELISA were:

15           Catching antibody A033 anti- $\alpha_2$ M,  
            Peroxidase-conjugated anti- $\alpha_2$ M antibody PE326,  
            1,2-Phenylenediamine, dihydrochloride (OPD)  
            all from DAKOPATTS A/S, Copenhagen, Denmark.  
            Urea peroxide, 125 mg, was from Organon Teknika.  
20           96 well ELISA plates were from NUNC, Copenhagen.

Coating buffer:

100 mM carbonate buffer pH 9.6 was made up as follows:  
Add 3.18 g  $\text{Na}_2\text{CO}_3$  and 5.96 g  $\text{NaHCO}_3$  to 1000 ml water.

25

Standard and sample buffer:

To 100 ml of 150 mM phosphate buffer pH 7.2 was added:  
50  $\mu$ l Tween 20  
2 g Bovine Serum Albumin (Sigma A 7030).

30

Washing buffer:

10 mM sodium phosphate pH 7.4  
145 mM sodium chloride  
0.1 % Tween 20.

35

Citric acid-phosphate buffer, pH 4.9:

The following reagents were added to 1000 ml of water  
7.3 g citric acid  
23.88 g  $\text{Na}_2\text{HPO}_4$ , 12  $\text{H}_2\text{O}$

0.5 ml Tween 20

The buffer was used for a maximum of 14 days, stored at 4°C.

Urea peroxide solution:

- 5            125 mg urea peroxide was dissolved in 8.93 ml water.  
The solution was kept in the dark at 4°C.

Coating of the plates for assay:

The 96 well plate was coated with 175  $\mu$ l of the DAKO A033 antibody diluted 1:1000 in the coating buffer. The plate was incubated over night at 4°C. Before use the plate was washed 4 times in washing buffer.

Application of standards and samples:

100  $\mu$ l standard or sample was added to each well. As a standard 15 purified human  $\alpha_2$ M, 2 mg/ml (prepared as described in: Sottrup-Jensen et al., (1983) Ann. N.Y. Acad. Sci. 421: 41-60) was used. The standard curve included the following serial dilutions: 1:4000, 1:8000, 1:16000 etc. down to 1:1024000, corresponding to final concentrations from 500  $\mu$ g/l down to 1.95  $\mu$ g/l. All dilutions were done in the Standard and sample buffer. The plate was incubated over night at 4°C and then washed 4 times with wash buffer before the next step.

Addition of conjugated antibody:

100  $\mu$ l of PE326, which had been diluted 1:6000 in the Standard and sample buffer, was added to each well. The plate was incubated for 2 h at 20°C, and then washed 4 times with wash buffer.

Enzyme activation:

8 mg of OPD was dissolved in 12 ml of Citric acid- phosphate buffer. To this solution 500  $\mu$ l Urea peroxide solution was added and the mixture was used immediately. 100  $\mu$ l of the final solution was added to each well, and the plate was incubated in the dark for 6 min. Then 100  $\mu$ l of 2 M  $H_2SO_4$  was added to each well and the  $A_{492}$  was read in an automated ELISA plate reader.

35

The above described ELISA did not give any background on medium supplemented with 10% FCS, nor did it give any background in BHK cell conditioned medium. Of 24 isolated MTX resistant clones, 16 produced detectable amounts of recombinant  $\alpha_2$ M.

Selected cell lines that secreted 12.3 mg/l (K16-6) and 19.1 mg/l (K17-6) in the supernatant (grown in a 6 well NUNC-plate) over a 48 hour period were expanded for large scale production of recombinant human  $\alpha_2$ M ( $\alpha_2$ M).

5

#### Purification of recombinant human $\alpha_2$ M.

Cell lines K16-6 and K17-6 were each expanded into one ten-double tray (NUNC, Denmark) with a growth surface of 6000 cm<sup>2</sup>. At 80% confluency the medium on the cells was changed from containing the 10% fetal 10 calf serum (FCS) down to 2%. After 48 hours of growth in medium with only 2% (FCS), the medium was removed, and the cells were washed twice with serum free medium. Cells were then grown serum free for 4 to 5 days with change of serum free medium every two days. Conditioned medium was pooled and analyzed for  $\alpha_2$ M by ELISA.

15

The pooled conditioned medium from K16-6 and from K17-6 contained 7.15 mg/l and 21.5 mg/l of  $\alpha_2$ M, respectively.

The  $\alpha_2$ M was purified according to published procedures (Sottrup-Jensen et al., (1983) Ann. N. Y. Acad. Sci. 421: 41-60). Briefly the conditioned medium was loaded onto a 10 ml Zn-Chelate column (Zn<sup>2+</sup>-iminodiacetic acid Sepharose 4B (Porath, J. et al., (1975) Nature 258: 598-599) equilibrated with 25 mM Tris-HCl pH 8.0, and washed with 100 ml phosphate buffered saline (PBS) pH 7.2 until  $A_{280} < 0.036$ . A second wash with 20 mM sodium phosphate, 500 mM NaCl pH 6.2 was performed until  $A_{280} < 0.033$ . The flow rate was 100 ml/hr and 3 ml fractions were collected.  $\alpha_2$ M was eluted 25 with 100 mM EDTA pH 7.0 at a flow rate of 40 ml/hr. During elution 1 ml fractions were collected.

Recovery of  $\alpha_2$ M was 44%. The  $\alpha_2$ M containing fractions were concentrated to 1 ml on an Amicon devise equipped with a PM 10 membrane and then loaded onto a Superose 12 gelfiltration column (25 mM Tris-HCl, 150 mM 30 NaCl pH 8.0). The  $\alpha_2$ M containing fractions were pooled and stored at -20°C until analysis.

#### EXAMPLE 3.

##### Characterization of recombinant human $\alpha_2$ M.

35

A. Chemical reactions at the thiol ester: thermal fragmentation and methylamine induced cleavage.



A number of different analyses were performed to evaluate the structural and biological characteristics of the human  $\alpha_2$ M as compared to a preparation of human plasma derived  $\alpha_2$ M, designated preparation LSJ39.

An important structural feature of  $\alpha_2$ M is the presence of the 5 thiol ester. When heated to 95°C for 15 min, the thiol ester will induce a peptide bond cleavage in the backbone of  $\alpha_2$ M at the position of the thiol esterified Glx-residue. This results in the fragmentation of the 180 kD  $\alpha_2$ M monomer into two polypeptides of 120 kD and 60 kD. Fig. 3 shows an analysis of both the purified  $\alpha_2$ M (from two transformed BHK cell lines) and the 10 purified human plasma derived preparation LSJ39 on a 10-20% SDS polyacrylamide gel. The different preparations, either native human or BHK cell derived recombinant  $\alpha_2$ M were all heat treated to induce thermal fragmentation before loading onto the gel. Molecular weight markers (from top to bottom: 180, 120, 92, 60, 43, 26, 14 and 6 kD) were applied to lanes 1 and 158. Samples in lanes 2, 3 and 4 were not reduced before electrophoresis, while samples in lanes 5, 6 and 7 were reduced. Preparation LSJ39 was applied to lanes 2 and 5.  $\alpha_2$ M K16-6 was applied to lanes 3 and 6, and  $\alpha_2$ M K17-6 was applied to lanes 4 and 7.

It was clear from the patterns of protein fragments on the gel, 20 that both human  $\alpha_2$ M and the two  $\alpha_2$ M preparations showed a considerable degree of thermal fragmentation. As expected, only the reduced samples displayed this fragmentation. In the nonreduced samples, the molecules migrated as the 360 kD dimer.

In the human plasma derived preparation LSJ39 (lane 5) a fragment 25 migrating slightly faster than the 60 kD fragment could be observed. Lanes 6 and 7 indicated the presence in the recombinant material of a similar faster migrating fragment. It is possible that this fragment represented a slightly underglycosylated variant of the 60 kD fragment.

Methylamine (MA) and other small nitrogen containing nucleophilic 30 will cleave the thiol ester and thereby inactivate the ester (Sottrup-Jensen, L., et al., (1980) FEBS Lett. 121: 275-280; Salvesen, G.S. et al., (1981) Biochem. J. 195: 453-461). After MA induced inactivation of the thiol ester, thermal fragmentation of  $\alpha_2$ M can no longer be observed.

Fig. 4 shows a SDS-PAGE run similar to that shown in Fig. 3 (with 35 respect to loaded samples), in which applied  $\alpha_2$ M and  $\alpha_2$ M had been pretreated with MA. From this gel it was concluded, that the thiol ester of  $\alpha_2$ M was just as susceptible to cleavage with MA as the thiol ester of native  $\alpha_2$ M. Upon reduction MA-treated  $\alpha_2$ M and  $\alpha_2$ M migrated as a single 180 kD monomer species.

Lanes 5 of both Fig. 3 and 4 showed an additional band of approximately 85 kD. When  $\alpha_2$ M is cleaved in the bait region by proteinases present in the blood, it generates two fragments, each with a molecular weight of 85 kD. The human  $\alpha_2$ M preparation LSJ39 (purified from serum) 5 contained these cleavage products, while they could not be detected on this gel in the two  $\alpha_2$ M preparations. This indicated that the material secreted from the transformed BHK cell lines was largely native uncomplexed  $\alpha_2$ M. Any  $\alpha_2$ M molecules, that have reacted with proteinases are inactivated and can not form additional complexes with other proteinases. Since the BHK cell 10 does not produce any proteinases that forms complexes with the  $\alpha_2$ M product, this cell is therefore well suited for production of recombinant human  $\alpha_2$ M.

#### B. Reaction with trypsin.

Reaction with trypsin is a standard way of analyzing the proteinase-complex 15 formation ability of  $\alpha_2$ M (Sottrup-Jensen, L. (1987) in: "The Plasma Proteins" (Putnam, F.W., ed.) 2nd Ed., 5: 191-291, Academic Press, Orlando, FL; Harpel, P.C. (1973) J. Exp. Med. 138: 508-521; Harpel, P.C., et al., (1979) J. Biol. Chem. 254: 8869-8878; Swenson, R.P. and Howard, J.B. (1979) J. Biol. Chem. 254: 4452-4456). In this reaction trypsin will cleave at its target site(s) 20 in the bait region of  $\alpha_2$ M, and the resulting reduced cleavage products (85 kD) will migrate as a double band. Under nonreducing conditions the trypsin- $\alpha_2$ M complexes will migrate as high molecular weight products.

Fig. 5 shows the result of such an analysis (performed as described (Sottrup-Jensen, L. (1987) in: "The Plasma Proteins" (Putnam, F.W., 25 ed.) 2nd Ed., 5: 191-291, Academic Press, Orlando, FL; Harpel, P.C. (1973) J. Exp. Med. 138: 508-521; Harpel, P.C., et al., (1979) J. Biol. Chem. 254: 8869-8878; Swenson, R.P. and Howard, J.B. (1979) J. Biol. Chem. 254: 4452-4456)) on the native human  $\alpha_2$ M preparation LSJ39 (lanes 2 and 5) and on  $\alpha_2$ M from cell lines K16-6 (lanes 3 and 6) and K17-6 (lanes 4 and 7). The samples 30 in lanes 2, 3 and 4 were not reduced before electrophoresis, while the samples in lanes 5, 6 and 7 were. Lane 5 shows that almost all of the human native  $\alpha_2$ M was cleaved with trypsin, while the two preparations of  $\alpha_2$ M were cleaved with an efficiency of approximately 80% or more. Without reduction of the complexes no low molecular weight products from the reaction between 35 trypsin and the native  $\alpha_2$ M or the BHK cell derived  $\alpha_2$ M were seen on the gel. The 85 kD fragments derived from the recombinant material migrated somewhat faster than the human standard; as mentioned above the recombinant material might be slightly underglycosylated.

When  $\alpha_2M$  is reacted with methylamine, the thiol ester will be inactivated, and  $\alpha_2M$  changes conformation from the "slow" form to the "fast" form (Sottrup-Jensen, L. (1987) in: The Plasma Proteins (Putnam, F.W., ed.) 2nd Ed., 5: 191-291, Academic Press, Orlando, FL; Van Leuven, F., Cassiman, J.-J. and Van Den Berghe, H. (1981) J. Biol. Chem. 256: 9016-9022). In this conformation it can no longer react rapidly with or form complexes with proteinases such as e.g. trypsin.

Fig. 6 shows the results of a set of experiments that were run in parallel to the experiments described above and shown in Fig. 5. However, before reaction with trypsin the native human  $\alpha_2M$  and the  $r\alpha_2M$  used in this experiment had been treated with methylamine (Sottrup-Jensen, L., et al., (1980) FEBS Lett. 121: 275-280). Under these conditions both the native  $\alpha_2M$  and the  $r\alpha_2M$  show a marked decrease in reactivity towards trypsin (80% or more of the  $\alpha_2M$  and  $r\alpha_2M$  monomers were migrating as a 180 kD polypeptide). This indicates that trypsin does not rapidly cleave at the bait region in methylamine treated human  $\alpha_2M$  or in BHK cell derived  $r\alpha_2M$ .

In these types of experiments BHK cell derived  $r\alpha_2M$  has shown characteristics similar to those of native human  $\alpha_2M$ .

#### 20 C. Trypsin and methylamine induced conformational change in $\alpha_2M$ .

As mentioned above the  $\alpha_2M$  molecule will undergo a conformational change both through complex formation with proteinases and through methylamine induced cleavage of the thiol ester. The change in structure results in an altered mobility on rate gels (Sottrup-Jensen, L. (1987) in: The Plasma Proteins (Putnam, F.W., ed.) 2nd Ed., 5: 191-291, Academic Press, Orlando, FL; Van Leuven, F., Cassiman, J.-J. and Van Den Berghe, H. (1981) J. Biol. Chem. 256: 9016-9022); unreacted  $\alpha_2M$  will migrate as a "slow" form, while reacted  $\alpha_2M$  will migrate as a "fast" form.

Fig. 7 and Fig. 8 show these conformational changes, as they appear after reaction with trypsin and methylamine, respectively (analyzed on 5-10% rate gels).

Lanes 1 on both gels contain purified human pregnancy zone protein (PZP) (Sand, O. et al.; (1985) J. Biol. Chem. 260: 15723-15735), which is known to appear in both a dimeric (D) and a tetrameric (T) configuration.

Lanes 2 on both gels contain unreacted human  $\alpha_2M$  preparation LSJ39. Lanes 3 on both gels show the fast migrating form, resulting from reaction with trypsin and methylamine, respectively. Lanes 4 on both gels show the unreacted  $r\alpha_2M$  preparation K16-6, and lanes 5 show the corresponding

fast forms. Lanes 6 on both gels show the unreacted  $\alpha_2$ M preparation K17-6, and lanes 7 show the corresponding fast forms.

It can be concluded that both complex formation between  $\alpha_2$ M and trypsin and reaction of  $\alpha_2$ M with methylamine result in the appearance of 5 fast migrating structures. These structures appear (as analyzed on rate gels) to be very similar to the structures obtained when human  $\alpha_2$ M was allowed to react with trypsin and methylamine. It is also evident from these figures that the  $\alpha_2$ M proteins showed a migration, which, when compared to the migration of dimeric and tetrameric PZP on the gels, is in agreement with the finding that these molecules are produced and secreted from the BHK cells in the active tetrameric conformation.

#### D. Chromatography of $\alpha_2$ M on a Superose 6 column.

A Superose 6 column can partially resolve  $\alpha_2$ M molecules in the dimeric configuration from molecules in the tetrameric configuration (Sottrup-Jensen, L. unpublished). Human standard  $\alpha_2$ M and  $\alpha_2$ M was analyzed on a 24 ml Superose 6 column (buffer: 25 mM Tris-HCl, 125 mM NaCl pH 8.0; flow rate: 1 ml/min; fraction size: 1 ml). Fig. 9 shows the diagrams obtained from the chromatography of purified human standard  $\alpha_2$ M and  $\alpha_2$ M from the K17-206 and the K16-6 BHK cell lines. Tetrameric  $\alpha_2$ M (Sottrup-Jensen, unpublished observation) will elute in fraction 12 on this type of column. It is evident from the chromatograms that both of the  $\alpha_2$ M preparations eluted in fraction 12, as did the human standard  $\alpha_2$ M. On this type of column, dimeric  $\alpha_2$ M molecules will elute in fraction 14 and 15 (Sottrup-Jensen, unpublished observation). This type of analysis supported the results obtained from the rate gels (Figs. 7 and 8), that  $\alpha_2$ M was secreted from BHK cells in a tetrameric configuration.

#### E. Trypsin protection analysis.

When trypsin is trapped inside the  $\alpha_2$ M molecule, it retains its catalytic capacity towards low molecular weight substrates such as S-2222 (N-benzoyl-L-Ile-L-Glu-Gly-L-Arg-p-nitroanilide). If trypsin is efficiently complexed with  $\alpha_2$ M, it will be protected against high molecular weight inhibitors such as Soybean Trypsin Inhibitor (STI) (Sottrup-Jensen, L. (1987) in: The Plasma Proteins (Putnam, F.W., ed.) 2nd Ed., 5: 191-291, Academic Press, Orlando, FL; Ganrot, P.O. (1966) Clin. Chim. Acta. 14: 493-501; Sottrup-Jensen, L. et al., (1981) FEBS Lett. 128: 127-132).

K16-6 and K17-6 derived  $\alpha_2$ M was compared with human plasma  $\alpha_2$ M in such a protection assay. 100  $\mu$ l  $\alpha_2$ M (in 25 mM Tris-HCl, 125 mM NaCl, pH

8.0) was mixed with 30  $\mu$ l trypsin (0.5 mg/ml in 20 mM sodium acetate pH 5.0). After incubating for 2 min. 30  $\mu$ l 1 mg/ml STI (in PBS) was added. 10  $\mu$ l aliquots were removed after 2 and 4 min. and each mixed with 750  $\mu$ l 0.12 mM S-2222 (dissolved 0.1 M sodiumphosphate pH 8.0, 5% dimethylsulfoxide).

5 The change in absorbance at 405 nm was recorded for 2 min. The results of the assay are given in the following Table II:

TABLE II

10	Prep. of $\alpha_2$ M.	$\alpha_2$ M in cuvette.		Activity.
		$A_{405}/\text{min}$	$\mu\text{g}$	
15	Human LSJ39	0.140	5.00	0.028
	K16-6	0.111	4.62	0.024
	K17-6	0.119	4.87	0.024

20

From these results it can be concluded that  $\alpha_2$ M had essentially the same protection capacity for trypsin against STI as compared with the protection capacity of human plasma  $\alpha_2$ M.

If  $\alpha_2$ M is treated with methylamine before the protection assay, 25 the protection capacity drops dramatically. In a similar assay as that described above, methylamine treated human plasma  $\alpha_2$ M only retained 17% of its protection capacity, while K16-6 and K17-6  $\alpha_2$ M retained 16% and 14% respectively. It can be concluded that  $\alpha_2$ M protected trypsin against STI with almost the same efficiency as did human plasma  $\alpha_2$ M.

30

#### E. Amino terminal amino acid sequencing of $\alpha_2$ M.

Theoretically, the  $\alpha_2$ M characterized in the present investigation could only be either bovine (contaminant from serum), from hamster (endogenous product from the BHK cell) or derived from expression of the 35 transfected plasmid p1167. The ELISA assay used never recognized any  $\alpha_2$ M in BHK cell conditioned medium, whether with or without added fetal calf serum. To make sure that the investigated  $\alpha_2$ M was human  $\alpha_2$ M, and to characterize the amino terminal processing of the recombinant product, amino terminal amino acid sequence determination was carried on out K16-6 and K17-6  $\alpha_2$ M as 40 described (Sottrup-Jensen, L. et al., (1984) J. Biol. Chem. 259: 8293-8303). The Edman degradation was repeated for 12 cycles, and the identity of the detected amino acid derivative in each cycle, was in total agreement with the

amino terminal sequence of human  $\alpha_2$ M: Ser-Val-Ser-Gly-Lys-Pro-Gln-Tyr-Met-Val-Leu-Val-, whereas bovine  $\alpha_2$ M has the following amino terminal sequence: Ala-Val-Asp-Gly-Lys-Pro-Gln-Tyr-Met-Val-Leu-Val- (unpublished, Dr. Torsten Kristensen, Department of Molecular Biology, University of Aarhus, Denmark.)

5

#### EXAMPLE 4.

##### Construction and expression of a bait region mutant of human $\alpha_2$ M.

In the present example it is demonstrated that the bait region of human  $\alpha_2$ M can be substituted by the bait region of human pregnancy zone protein (PZP) (Sottrup Jensen, L., Folkersen, J., Kristensen, T. and Tack, B.F. Partial primary structure of human pregnancy zone protein: extensive sequence homology with human alpha 2-macroglobulin. Proc. Natl. Acad. Sci. U.S.A. 81, 7353-7357, 1984; Sand, O., Folkersen, J., Westergaard, J.G. and Sottrup Jensen, L. Characterization of human pregnancy zone protein. Comparison with human alpha 2-macroglobulin. J.Biol.Chem. 260, 15723-15735, 1985). The resulting  $\alpha_2$ M bait region mutant exhibited a proteinase inhibitor profile similar to that of human pregnancy zone protein.

To facilitate substitution of DNA fragments encoding the bait region of human  $\alpha_2$ M cDNA, target sites for the restriction enzymes PstI and SacII were introduced at the 5' and at the 3' end of the cDNA region encoding the bait region.

The human  $\alpha_2$ M expression plasmid p1167 was digested with BamHI and ClaI, and a 2660 bp fragment, which carried the central part of the human  $\alpha_2$ M cDNA, was subcloned in the BamHI and ClaI digested vector pSX191.

This vector, which had previously been constructed, is a derivative of pUC19. It was constructed as described: pUC19 was digested with EcoRI and HindIII, and a synthetic linker with the following sequence

```

      KpnI  PstI  EcoRI Hind3 ClaI  SphI  BamHI
30 AATTGGTACCCTGCAGGAATTCAAGCTTATCGATGGCATGCGGATCC - NOR781
    CCATGGGACGTCCTTAAGTTCGAATAGCTACCGTACGCCTAGGTCGA - NOR782
  
```

was cloned in the digested pUC19 vector. The linker, which was an annealing product from the two synthetic oligonucleotides NOR781 and NOR782, has cohesive ends that will ligate to the EcoRI and the HindIII sites of pUC19 in such a way that these ligation sites are not regenerated in the pSX191 vector. Thus pSX191 carried sites for KpnI, PstI, EcoRI, HindIII, ClaI, SphI and BamHI.

The resulting plasmid pSX191 $\alpha_2$ M was digested with BamHI and HindIII, and a purified 2.6 kb BamHI/HindIII  $\alpha_2$ M fragment was cloned in

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M13mp18 to generate M13mp18 $\alpha_2$ M for mutagenesis by described methods. A synthetic oligonucleotide NOR973, with the following sequence:

5'(TTCATACTGCTGCAGCTGTGGACAC)3'

was used to introduce a PstI site at position 2102 (SEQ ID NO:1) in the cDNA 5 sequence, and a oligonucleotide (NOR974) with the following sequence:

5'(AGCCACCCCGCGGAGTTTACCAC)3'

was used to introduce a SacII site at position 2271 (SEQ ID NO:1) in the cDNA sequence. These sites were chosen because they did not introduce alterations in the encoded amino acid sequence, and they were within a 10 convenient distance of the bait region in human  $\alpha_2$ M cDNA. Both primers were used in the same mutagenesis experiment (Kunkel, T.A., Roberts, J.D. and Zakour, R.A. Rapid and Efficient Site-Specific Mutagenesis without Phenotypic Selection. Methods in Enzymol. 154, 367-382, 1987); dsDNA was isolated from mutated M13mp18 $\alpha_2$ M plaques, and the DNA was digested with the restriction 15 enzymes PstI and SacII. Correctly mutated recombinants, which had an insert of 160 bp, were further analyzed by DNA sequencing (Tabor, S. and Richardson, C.C. DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. Proc. Natl. Acad. Sci. U.S.A. 84, 4767-4771, 1987). A 2.6 kb BamHI/HindIII fragment from a correct  $\alpha_2$ M cDNA mutant (M13mp18 $\alpha_2$ M#212.1) was subcloned in 20 a BamHI/HindIII digested pUC13 vector, and a correct subclone p1308 was isolated and characterized with BamHI/HindIII and PstI/SacII double digestions and DNA electrophoresis.

The PstI/SacII fragment in p1308 can be excised and replaced with a different DNA fragment, which encodes bait region variants. The 25 resulting new variants (bait region mutants or analogs) of  $\alpha_2$ M cDNA can be isolated as BamHI/ClaI fragments and subcloned back into BamHI/ClaI digested expression vector p1167.

In the present example DNA encoding the amino acids of the bait region for human PZP (Sottrup-Jensen et al. 1989, supra) was obtained from 30 ligation, annealing and cloning of 8 synthetic oligonucleotides.

The DNA sequence of the synthetic fragment and the encoded amino acids as inserted into the  $\alpha_2$ M clone are given in SEQ ID NO:3, and comprises positions 2107 to 2305 and the corresponding amino acids. A PstI site was introduced at the 5' end in the synthetic fragment, and SacII and BamHI sites 35 were introduced at the 3' end.

This synthetic 0.2 kb DNA fragment was cloned in a PstI/BamHI digested M13mp18 vector for DNA sequencing. DNA from a clone containing the correct sequence was digested with PstI and SacII, and the purified 0.2 kb fragment was cloned in a PstI/SacII digested and gel purified p1308 vector.

A correct recombinant, p267PZP, was characterized with restriction enzyme digestions, and from this plasmid, bait region mutated ( $\alpha_2M \rightarrow PZP$ ) cDNA was isolated as a 2.7 kb BamHI/ClaI fragment and subcloned in a BamHI/ClaI digested  $\alpha_2M$  expression vector p1167. The resulting plasmid, designated p1365, was grown as a large scale plasmid preparation, purified by CsCl centrifugation, and cotransfected with pDHFR-I into BHK cells.

Through this procedure the nucleotides 2102 to 2275 in SEQ ID NO:1 was removed and replaced with nucleotides 2102 to 2305 in SEQ ID NO:3.

The procedures for transfection, selection of bait region mutated  $\alpha_2M$  (designated  $\alpha_2M$ -PZP) recombinants (with an  $\alpha_2M$  specific ELISA), large scale production and purification of mutated  $\alpha_2M$  were as described elsewhere (EXAMPLE 2) in this application.

Characterization of the proteinase inhibitor specificity of a bait region  
15 mutant of human  $\alpha_2M$ .

The purified recombinant  $\alpha_2M$  mutant,  $\alpha_2M$ -PZP, was characterized with respect to its inhibitor specificity profile against various proteinases by the use of previously described methods (Sand et al.1985). For comparison human plasma derived  $\alpha_2M$  and PZP were treated with the same set  
20 of proteinases in parallel reactions. The proteinases used were chymotrypsin, elastase, trypsin and Staphylococcus aureus Glu-specific proteinase. It has been reported (Sand et al.1985) that chymotrypsin and elastase show a rapid reaction with both PZP and  $\alpha_2M$ , while the reaction between the two  
proteinase inhibitors and trypsin and Staphylococcus aureus Glu-specific  
25 proteinase is quite dissimilar for PZP and  $\alpha_2M$ : both proteinases react rapidly with  $\alpha_2M$ , while the reaction with PZP is slow (Sand et al.1985). The reason for this difference in reaction rate with the different proteinases is believed to be due to the fact that the bait region in PZP contains strong specificity determinant for chymotrypsin and elastase, but none for trypsin  
30 and Staphylococcus aureus Glu-specific proteinase.

The results of the analysis is presented in figures 10 to 13.

Figure 10 illustrates the gel electrophoresis (10 - 20 % reducing SDS-PAGE) of the reaction products from chymotrypsin treated human  $\alpha_2M$ , human PZP and  $\alpha_2M$ -PZP. Molecular weight markers (from top to bottom: 180, 120, 92,  
35 60, 43, 26, 14 and 6 kD) were applied to lanes 1 and 8. All samples were reduced. Lanes 2, 3 and 4 show the cleavage products obtained from reaction of chymotrypsin with human plasma derived PZP,  $\alpha_2M$ -PZP and human plasma derived  $\alpha_2M$ , respectively. The ratio of proteinase to inhibitor was 1:1. Lanes 5, 6 and 7 show cleavage products from similar reactions at a ratio of 2:1



between proteinase and the three tested inhibitors. In all 6 lanes cleavage products (85 kD) could be identified. This indicated that  $\alpha_2$ M-PZP reacted with chymotrypsin with similar characteristics as did human plasma derived  $\alpha_2$ M and PZP.

5 Figure 11 illustrates the gel electrophoresis (10 - 20 % reducing SDS-PAGE) of the reaction products from elastase treated human  $\alpha_2$ M, human PZP and  $\alpha_2$ M-PZP. Molecular weight markers were the same as applied on the gel in Fig. 2. All samples were reduced. Lanes 2, 3 and 4 show the cleavage products obtained from reaction of elastase with human plasma derived PZP, 10  $\alpha_2$ M-PZP and human plasma derived  $\alpha_2$ M, respectively. The ratio of proteinase to inhibitor was 1:1. Lanes 5, 6 and 7 show cleavage products from similar reactions at a ratio of 2:1 between proteinase and the three tested inhibitors. In all 6 lanes cleavage products (85 kD) could be identified. This indicated that  $\alpha_2$ M-PZP reacted with elastase with similar characteristics as did human plasma derived  $\alpha_2$ M and PZP.

Figure 12 illustrates the gel electrophoresis (10 - 20 % reducing SDS-PAGE) of the reaction products from trypsin treated human  $\alpha_2$ M, human PZP and  $\alpha_2$ M-PZP. Molecular weight markers were the same as applied on the gel in Fig. 2. All samples were reduced. Lanes 2, 3 and 4 show the cleavage 20 products obtained from reaction of trypsin with human plasma derived PZP, human plasma derived  $\alpha_2$ M and  $\alpha_2$ M-PZP, respectively. The ratio of proteinase to inhibitor was 1:1. Lanes 5, 6 and 7 show cleavage products from similar reactions at a ratio of 2:1 between proteinase and the three tested inhibitors. In lanes 3 and 6 cleavage products (85 kD) could be identified 25 from the reaction between trypsin and  $\alpha_2$ M. In lanes 2, 4, 5 and 7 no cleavage products were observed from the reaction of trypsin with PZP and  $\alpha_2$ M-PZP. This result demonstrated that  $\alpha_2$ M-PZP reacted poorly with trypsin as did human plasma derived PZP, while  $\alpha_2$ M was cleaved in the reaction with trypsin.

Figure 13 illustrates the gel electrophoresis (10 - 20 % reducing 30 SDS-PAGE) of the reaction products from Staphylococcus aureus Glu-specific protease treated human  $\alpha_2$ M, human PZP and  $\alpha_2$ M-PZP. Molecular weight markers were the same as applied on the gel in Fig. 2. All samples were reduced. Lanes 2, 3 and 4 show the cleavage products obtained from reaction of Staphylococcus aureus Glu-specific protease with human plasma derived PZP, 35  $\alpha_2$ M-PZP and human plasma derived  $\alpha_2$ M, respectively. The ratio of proteinase to inhibitor was 1:1. Lanes 5, 6 and 7 show cleavage products from similar reactions at a ratio of 2:1 between proteinase and the three tested inhibitors. In lanes 4 and 7 cleavage products (85 kD) could be identified from the reaction between Staphylococcus aureus Glu-specific protease and

$\alpha_2$ M. In lanes 2, 3, 5 and 6 much less cleavage product could be identified from the reaction of this proteinase with PZP and  $\alpha_2$ M-PZP. This result demonstrated that  $\alpha_2$ M-PZP reacted poorly with the Staphylococcus aureus proteinase as did human plasma derived PZP, while  $\alpha_2$ M was cleaved in the 5 reaction with this proteinase.

It can be concluded that  $\alpha_2$ M-PZP showed the same pattern of reaction with four proteinases as did human plasma derived PZP. This pattern of reaction was different from the corresponding pattern obtained from reaction with  $\alpha_2$ M. Thus  $\alpha_2$ M-PZP has been demonstrated to have a proteinase 10 inhibitor profile similar to native PZP and dissimilar to  $\alpha_2$ M. Thus it has been demonstrated that the proteinase inhibitor profile of  $\alpha_2$ M can be modulated by substitution of DNA fragments encoding the bait region.

The substitution as described in this invention did not destroy the activity of the proteinase inhibitor, and it is therefore demonstrated 15 that functional macroglobulin hybrids can be constructed by substitutions (mutations) in the bait region. The finding will lead to the design of  $\alpha_2$ M-derivatives with new desired proteinase specificities. No doubt, these results could be extended to other macroglobulin based hybrids, in which the bait region can be modified at will to obtain new inhibitor specificities.

20 Aggressive activity of proteinases is often a problem in relation to various diseases (e.g. the activity of elastase and cathepsin G in severe inflammation leads to tissue and organ destruction and failure). Inhibitors of such proteinases will be useful in drug design. In situations where the target site for the proteinase is known, but no inhibitor can be identified, 25  $\alpha_2$ M can be engineered (mutated in the bait region) to obtain the desired specificity. In a situation where the target specificity of the proteinase in question is unknown, saturation mutagenesis or random synthesis of the bait region will lead to an indefinite number of target sequences that can be introduced and expressed in hybrid macroglobulins. These hybrids can be 30 screened for proteinase inhibition, and the target sequence(s) can be identified. The resulting  $\alpha_2$ M analog can be produced and purified as described elsewhere in this invention. Upon injection into the circulation such  $\alpha_2$ M analogs will inhibit and clear from the blood any proteinase of the given specificity.

35 Introduction of protein analogs or mutants in the human body always raises the possibility for antigenicity. The generation of a panel of 45 mouse monoclonal antibodies against human  $\alpha_2$ M has been described (Van Leuven et al.1988; Delain et al.1988). None of these antibodies were directed against the bait region. This indicates that the bait region is not highly

antigenic and that mutants in this region of the molecule can be generated and used for therapeutical uses without risk for antibody development.

## SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Novo Nordisk A/S
- (ii) TITLE OF INVENTION: Expression of Plasma Glycoproteins
- (iii) NUMBER OF SEQUENCES: 4
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Novo Nordisk A/S, Patent Department
  - (B) STREET: Novo Alle
  - (C) CITY: Bagsvaerd
  - (E) COUNTRY: DENMARK
  - (F) ZIP: DK-2880
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: DK 4235/89, DK 4236/89, DK 4237/89
  - (B) FILING DATE: 29-AUG-1989

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 4569 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: N
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: Homo sapiens
  - (F) TISSUE TYPE: Hepatic
  - (G) CELL TYPE: Hepatoblastoma
  - (H) CELL LINE: HepG2
- (ix) FEATURE:
  - (A) NAME/KEY: CDS
  - (B) LOCATION: 29..4450
  - (D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GTCTCCTCCA GCTCCTTCTT TCTGCAAC ATG GGG AAG AAC AAA CTC CTT CAT  
Met Gly Lys Asn Lys Leu Leu His  
1 5

52

CCA AGT CTG GTT CTT CTC CTC TTG GTC CTC CTG CCC ACA GAC GCC TCA  
Pro Ser Leu Val Leu Leu Leu Val Leu Leu Pro Thr Asp Ala Ser

100

35

GTC Val 25	TCT Ser	GGA Gly	AAA Lys	CCG Pro	CAG Gln 30	TAT Tyr	ATG Met	GTT Val	CTG Leu	GTC Val 35	CCC Pro	TCC Ser	CTG Leu	CTC Leu	CAC His 40	148
ACT Thr	GAG Glu	ACC Thr	ACT Thr	GAG Glu 45	AAG Lys	GGC Gly	TGT Cys	GTC Val 50	CTT Leu	CTG Leu	AGC Ser	TAC Tyr	CTG Leu	AAT Asn 55	GAG Glu	196
ACA Thr	GTG Val	ACT Thr	GTA Val 60	AGT Ser	GCT Ala	TCC Ser	TTG Leu	GAG Glu 65	TCT Ser	GTC Val	AGG Arg	GGA Gly	AAC Asn 70	AGG Arg	AGC Ser	244
CTC Leu	TTC Phe	ACT Thr 75	GAC Asp	CTG Leu	GAG Glu	GCG Ala	GAG Glu 80	AAT Asn	GAC Asp	GTA Val	CTC Leu	CAC His 85	TGT Cys	GTC Val	GCC Ala	292
TTC Phe	GCT Ala 90	GTC Val	CCA Pro	AAG Lys	TCT Ser	TCA Ser 95	TCC Ser	AAT Asn	GAG Glu	GAG Glu 100	GTA Val	ATG Met	TTC Phe	CTC Leu	ACT Thr	340
GTC Val 105	CAA Gln	GTG Val	AAA Lys	GGA Gly	CCA Pro 110	ACC Thr	CAA Gln	GAA Glu	TTT Phe	AAG Lys 115	AAG Lys	CGG Arg	ACC Thr	ACA Thr	GTG Val 120	388
ATG Met	GTT Val	AAG Lys	AAC Asn	GAG Glu 125	GAC Asp	AGT Ser	CTG Leu	GTC Val	TTT Phe 130	GTC Val	CAG Gln	ACA Thr	GAC Asp	AAA Lys 135	TCA Ser	436
ATC Ile	TAC Tyr	AAA Lys	CCA Pro 140	GGG Gly	CAG Gln	ACA Thr	GTG Val	AAA Lys 145	TTT Phe	CGT Arg	GTT Val	GTC Val	TCC Ser 150	ATG Met	GAT Asp	484
GAA Glu	AAC Asn	TTT Phe 155	CAC His	CCC Pro	CTG Leu	AAT Asn 160	GAG Glu	TTG Leu	ATT Ile	CCA Pro	CTA Leu 165	GTA Val	TAC Tyr	ATT Ile	CAG Gln	532
GAT Asp	CCC Pro 170	AAA Lys	GGA Gly	AAT Asn	CGC Arg	ATC Ile 175	GCA Ala	CAA Gln	TGG Trp	CAG Gln 180	AGT Ser	TTC Phe	CAG Gln	TTA Leu	GAG Glu	580
GGT Gly 185	GGC Gly	CTC Leu	AAG Lys	CAA Gln	TTT Phe 190	TCT Ser	TTT Phe	CCC Pro	CTC Leu	TCA Ser 195	TCA Ser	GAG Glu	CCC Pro	TTC Phe	CAG Gln 200	628
GGC Gly	TCC Ser	TAC Tyr	AAG Lys	GTG Val 205	GTG Val	GTA Val	CAG Gln	AAG Lys	AAA Lys 210	TCA Ser	GGT Gly	GGA Gly	AGG Arg	ACA Thr	GAG Glu 215	676
CAC His	CCT Pro	TTC Phe	ACC Thr 220	GTG Val	GAG Glu	GAA Glu	TTT Phe 225	GTT Val	CTT Leu	CCC Pro	AAG Lys	TTT Phe 230	GAA Glu	GTA Val	CAA Gln	724
GTA Val	ACA Thr	GTG Val 235	CCA Pro	AAG Lys	ATA Ile	ATC Ile	ACC Thr 240	ATC Ile	TTG Leu	GAA Glu	GAA Glu	GAG Glu 245	ATG Met	AAT Asn	GTA Val	772

36

TCA GTG TGT GGC CTA TAC ACA TAT GGG AAG CCT GTC CCT GGA CAT GTG Ser Val Cys Gly Leu Tyr Thr Tyr Gly Lys Pro Val Pro Gly His Val 250 255 260	820
ACT GTG AGC ATT TGC AGA AAG TAT AGT GAC GCT TCC GAC TGC CAC GGT Thr Val Ser Ile Cys Arg Lys Tyr Ser Asp Ala Ser Asp Cys His Gly 265 270 275 280	868
GAA GAT TCA CAG GCT TTC TGT GAG AAA TTC AGT GGA CAG CTA AAC AGC Glu Asp Ser Gln Ala Phe Cys Glu Lys Phe Ser Gly Gln Leu Asn Ser 285 290 295	916
CAT GGC TGC TTC TAT CAG CAA GTA AAA ACC AAG GTC TTC CAG CTG AAG His Gly Cys Phe Tyr Gln Gln Val Lys Thr Lys Val Phe Gln Leu Lys 300 305 310	964
AGG AAG GAG TAT GAA ATG AAA CTT CAC ACT GAG GCC CAG ATC CAA GAA Arg Lys Glu Tyr Glu Met Lys Leu His Thr Glu Ala Gln Ile Gln Glu 315 320 325	1012
GAA GGA ACA GTG GTG GAA TTG ACT GGA AGG CAG TCC AGT GAA ATC ACA Glu Gly Thr Val Val Glu Leu Thr Gly Arg Gln Ser Ser Glu Ile Thr 330 335 340	1060
AGA ACC ATA ACC AAA CTC TCA TTT GTG AAA GTG GAC TCA CAC TTT CGA Arg Thr Ile Thr Lys Leu Ser Phe Val Lys Val Asp Ser His Phe Arg 345 350 355 360	1108
CAG GGA ATT CCC TTC TTT GGG CAG GTG CGC CTA GTA GAT GGG AAA GGC Gln Gly Ile Pro Phe Phe Gly Gln Val Arg Leu Val Asp Gly Lys Gly 365 370 375	1156
GTC CCT ATA CCA AAT AAA GTC ATA TTC ATC AGA GGA AAT GAA GCA AAC Val Pro Ile Pro Asn Lys Val Ile Phe Ile Arg Gly Asn Glu Ala Asn 380 385 390	1204
TAT TAC TCC AAT GCT ACC ACG GAT GAG CAT GGC CTT GTA CAG TTC TCT Tyr Tyr Ser Asn Ala Thr Thr Asp Glu His Gly Leu Val Gln Phe Ser 395 400 405	1252
ATC AAC ACC ACC AAT GTT ATG GGT ACC TCT CTT ACT GTT AGG GTC AAT Ile Asn Thr Thr Asn Val Met Gly Thr Ser Leu Thr Val Arg Val Asn 410 415 420	1300
TAC AAG GAT CGT AGT CCC TGT TAC GGC TAC CAG TGG GTG TCA GAA GAA Tyr Lys Asp Arg Ser Pro Cys Tyr Gly Tyr Gln Trp Val Ser Glu Glu 425 430 435 440	1348
CAC GAA GAG GCA CAT CAC ACT GCT TAT CTT GTG TTC TCC CCA AGC AAG His Glu Glu Ala His His Thr Ala Tyr Leu Val Phe Ser Pro Ser Lys 445 450 455	1396
AGC TTT GTC CAC CTT GAG CCC ATG TCT CAT GAA CTA CCC TGT GGC CAT Ser Phe Val His Leu Glu Pro Met Ser His Glu Leu Pro Cys Gly His 460 465 470	1444

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ACT Thr	CAG Gln	ACA Thr 475	GTC Val	CAG Gln	GCA Ala	CAT His 480	TAT Tyr	ATT Ile	CTG Leu	AAT Asn	GGA Gly 485	GGC Gly	ACC Thr	CTG Leu	CTG Leu	1492
GGG Gly	CTG Leu 490	AAG Lys	AAG Lys	CTC Leu	TCC Ser	TTC Phe 495	TAT Tyr	TAT Tyr	CTG Leu	ATA Ile	ATG Met 500	GCA Ala	AAG Lys	GGA Gly	GGC Gly	1540
ATT Ile 505	GTC Val	CGA Arg	ACT Thr	GGG Gly 510	ACT Thr	CAT His	GGA Gly	CTG Leu	CTT Leu	GTG Val 515	AAG Lys	CAG Gln	GAA Glu	GAC Asp	ATG Met 520	1588
AAG Lys	GGC Gly	CAT His	TTT Phe	TCC Ser 525	ATC Ile	TCA Ser	ATC Ile	CCT Pro	GTG Val 530	AAG Lys	TCA Ser	GAC Asp	ATT Ile	GCT Ala	CCT Pro	1636
GTC Val	GCT Ala	CGG Arg	TTG Leu 540	CTC Leu	ATC Ile	TAT Tyr	GCT Ala	GTT Val 545	TTA Leu	CCT Pro	ACC Thr	GGG Gly 550	GAC Asp	GTG Val	ATT Ile	1684
GGG Gly	GAT Asp	TCT Ser 555	GCA Ala	AAA Lys	TAT Tyr	GAT Asp	GTT Val 560	GAA Glu	AAT Asn	TGT Cys	CTG Leu 565	GCC Ala	AAC Asn	AAG Lys	GTG Val	1732
GAT Asp	TTG Leu 570	AGC Ser	TTC Phe	AGC Ser	CCA Pro	TCA Ser 575	CAA Gln	AGT Ser	CTC Leu	CCA Pro	GCC Ala 580	TCA Ser	CAC His	GCC Ala	CAC His	1780
CTG Leu 585	CGA Arg	GTC Val	ACA Thr	GCG Ala	GCT Ala 590	CCT Pro	CAG Gln	TCC Ser	GTC Val	TGC Cys 595	GCC Ala	CTC Leu	CGT Arg	GCT Ala	GTG Val 600	1828
GAC Asp	CAA Gln	AGC Ser	GTG Val 605	CTG Leu	CTC Leu	ATG Met	AAG Lys	CCT Pro	GAT Asp 610	GCT Ala	GAG Glu	CTC Leu	TCG Ser	GCG Ala	TCC Ser 615	1876
TCG Ser	GTT Val	TAC Tyr	ACC Asn 620	CTG Leu	CTA Leu	CCA Pro	GAA Glu	AAG Lys 625	GAC Asp	CTC Leu	ACT Thr	GGC Gly 630	TTC Phe	CCT Pro	GGG Gly	1924
CCT Pro	TTG Leu	AAT Asn 635	GAC Asp	CAG Gln	GAC Asp	GAT Asp	GAA Glu 640	GAC Asp	TGC Cys	ATC Ile	AAT Asn 645	CGT Arg	CAT His	AAT Asn	GTC Val	1972
TAT Tyr	ATT Ile 650	AAT Asn	GGA Gly	ATC Ile	ACA Thr	TAT Tyr 655	ACT Thr	CCA Pro	GTA Val	TCA Ser	AGT Ser 660	ACA Thr	AAT Asn	GAA Glu	AAG Lys	2020
GAT Asp 665	ATG Met	TAC Tyr	AGC Ser	TTC Phe	CTA Leu 670	GAG Glu	GAC Asp	ATG Met	GGC Gly 675	TTA Leu	AAG Lys	GCA Ala	TTC Phe	ACC Thr	AAC Asn 680	2068
TCA Ser	AAG Lys	ATT Ile	CGT Arg	AAA Lys 685	CCC Pro	AAA Lys	ATG Met	TGT Cys	CCA Pro 690	CAG Gln	CTT Leu	CAA Gln	CAG Gln	TAT Tyr 695	GAA Glu	2116

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ATG CAT GGA CCT GAA GGT CTA CGT GTA GGT TTT TAT GAG TCA GAT GTA Met His Gly Pro Glu Gly Leu Arg Val Gly Phe Tyr Glu Ser Asp Val 700 705 710	2164
ATG GGA AGA GGC CAT GCA CGC CTG GTG CAT GTT GAA GAG CCT CAC ACG Met Gly Arg Gly His Ala Arg Leu Val His Val Glu Glu Pro His Thr 715 720 725	2212
GAG ACC GTA CGA AAG TAC TTC CCT GAG ACA TGG ATC TGG GAT TTG GTG Glu Thr Val Arg Lys Tyr Phe Pro Glu Thr Trp Ile Trp Asp Leu Val 730 735 740	2260
GTG GTA AAC TCA GCA GGT GTG GCT GAG GTA GGA GTA ACA GTC CCT GAC Val Val Asn Ser Ala Gly Val Ala Glu Val Gly Val Thr Val Pro Asp 745 750 755 760	2308
ACC ATC ACC GAG TGG AAG GCA GGG GCC TTC TGC CTG TCT GAA GAT GCT Thr Ile Thr Glu Trp Lys Ala Gly Ala Phe Cys Leu Ser Glu Asp Ala 765 770 775	2356
GGA CTT GGT ATC TCT TCC ACT GCC TCT CTC CGA GCC TTC CAG CCC TTC Gly Leu Gly Ile Ser Ser Thr Ala Ser Leu Arg Ala Phe Gln Pro Phe 780 785 790	2404
TTT GTG GAG CTT ACA ATG CCT TAC TCT GTG ATT CGT GGA GAG GCC TTC Phe Val Glu Leu Thr Met Pro Tyr Ser Val Ile Arg Gly Glu Ala Phe 795 800 805	2452
ACA CTC AAG GCC ACG GTC CTA AAC TAC CTT CCC AAA TGC ATC CGG GTC Thr Leu Lys Ala Thr Val Leu Asn Tyr Leu Pro Lys Cys Ile Arg Val 810 815 820	2500
AGT GTG CAG CTG GAA GCC TCT CCC GCC TTC CTA GCT GTC CCA GTG GAG Ser Val Gln Leu Glu Ala Ser Pro Ala Phe Leu Ala Val Pro Val Glu 825 830 835 840	2548
AAG GAA CAA GCG CCT CAC TGC ATC TGT GCA AAC GGG CGG CAA ACT GTG Lys Glu Gln Ala Pro His Cys Ile Cys Ala Asn Gly Arg Gln Thr Val 845 850 855	2596
TCC TGG GCA GTA ACC CCA AAG TCA TTA GGA AAT GTG AAT TTC ACT GTG Ser Trp Ala Val Thr Pro Lys Ser Leu Gly Asn Val Asn Phe Thr Val 860 865 870	2644
AGC GCA GAG GCA CTA GAG TCT CAA GAG CTG TGT GGG ACT GAG GTG CCT Ser Ala Glu Ala Leu Glu Ser Gln Glu Leu Cys Gly Thr Glu Val Pro 875 880 885	2692
TCA GTT CCT GAA CAC GGA AGG AAA GAC ACA GTC ATC AAG CCT CTG TTG Ser Val Pro Glu His Gly Arg Lys Asp Thr Val Ile Lys Pro Leu Leu 890 895 900	2740
GTT GAA CCT GAA GGA CTA GAG AAG GAA ACA ACA TTC AAC TCC CTA CTT Val Glu Pro Glu Gly Leu Glu Lys Glu Thr Thr Phe Asn Ser Leu Leu 905 910 915 920	2788



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TGT Cys	CCA Pro	TCA Ser	GGT Gly	GGT Gly	GAG Glu	GTT Val	TCT Ser	GAA Glu	GAA Glu	TTA Leu	TCC Ser	CTG Leu	AAA Lys	CTG Leu	CCA Pro	2836
			925					930						935		
CCA Pro	AAT Asn	GTG Val	GTA Val	GAA Glu	GAA Glu	TCT Ser	GCC Ala	CGA Arg	GCT Ala	TCT Ser	GTC Val	TCA Ser	GTT Val	TTG Leu	GGA Gly	2884
			940					945						950		
GAC Asp	ATA Ile	TTA Leu	GGC Gly	TCT Ser	GCC Ala	ATG Met	CAA Gln	AAC Asn	ACA Thr	CAA Gln	AAT Asn	CTT Leu	CTC Leu	CAG Gln	ATG Met	2932
		955					960					965				
CCC Pro	TAT Tyr	GGC Gly	TGT Cys	GGA Gly	GAG Glu	CAG Gln	AAT Asn	ATG Met	GTC Val	CTC Leu	TTT Phe	GCT Ala	CCT Pro	AAC Asn	ATC Ile	2980
	970					975					980					
TAT Tyr	GTA Val	CTG Leu	GAT Asp	TAT Tyr	CTA Leu	AAT Asn	GAA Glu	ACA Thr	CAG Gln	CAG Gln	CTT Leu	ACT Thr	CCA Pro	GAG Glu	ATC Ile	3028
	985				990					995					1000	
AAG Lys	TCC Ser	AAG Lys	GCC Ala	ATT Ile	GGC Gly	TAT Tyr	CTC Leu	AAC Asn	ACT Thr	GGT Gly	TAC Tyr	CAG Gln	AGA Arg	CAG Gln	TTG Leu	3076
				1005					1010					1015		
AAC Asn	TAC Tyr	AAA Lys	CAC His	TAT Tyr	GAT Asp	GGC Gly	TCC Ser	TAC Tyr	AGC Ser	ACC Thr	TTT Phe	GGG Gly	GAG Glu	CGA Arg	TAT Tyr	3124
			1020					1025					1030			
GGC Gly	AGG Arg	AAC Asn	CAG Gln	GGC Gly	AAC Asn	ACC Thr	TGG Trp	CTC Leu	ACA Thr	GCC Ala	TTT Phe	GTT Val	CTG Leu	AAG Lys	ACT Thr	3172
		1035					1040					1045				
TTT Phe	GCC Ala	CAA Gln	GCT Ala	CGA Arg	GCC Ala	TAC Tyr	ATC Ile	TTC Phe	ATC Ile	GAT Asp	GAA Glu	GCA Ala	CAC His	ATT Ile	ACC Thr	3220
	1050					1055					1060					
CAA Gln	GCC Ala	CTC Leu	ATA Ile	TGG Trp	CTC Leu	TCC Ser	CAG Gln	AGG Arg	CAG Gln	AAG Lys	GAC Asp	AAT Asn	GGC Gly	TGT Cys	TTC Phe	3268
	1065				1070					1075					1080	
AGG Arg	AGC Ser	TCT Ser	GGG Gly	TCA Ser	CTG Leu	CTC Leu	AAC Asn	AAT Asn	GCC Ala	ATA Ile	AAG Lys	GGA Gly	GGA Gly	GTA Val	GAA Glu	3316
				1085					1090					1095		
GAT Asp	GAA Glu	GTG Val	ACC Thr	CTC Leu	TCC Ser	GCC Ala	TAT Tyr	ATC Ile	ACC Thr	ATC Ile	GCC Ala	CTT Leu	CTG Leu	GAG Glu	ATT Ile	3364
			1100					1105				1110				
CCT Pro	CTC Leu	ACA Thr	GTC Val	ACT Thr	CAC His	CCT Pro	GTT Val	GTC Val	CGC Arg	AAT Asn	GCC Ala	CTG Leu	TTT Phe	TGC Cys	CTG Leu	3412
		1115					1120					1125				
GAG Glu	TCA Ser	GCC Ala	TGG Trp	AAG Lys	ACA Thr	GCA Ala	CAA Gln	GAA Glu	GGG Gly	GAC Asp	CAT His	GGC Gly	AGC Ser	CAT His	GTA Val	3460
	1130					1135					1140					

TAT ACC AAA GCA CTG CTG GCC TAT GCT TTT GCC CTG GCA GGT AAC CAG Tyr Thr Lys Ala Leu Leu Ala Tyr Ala Phe Ala Leu Ala Gly Asn Gln 1145 1150 1155 1160	3508
GAC AAG AGG AAG GAA GTA CTC AAG TCA CTT AAT GAG GAA GCT GTG AAG Asp Lys Arg Lys Glu Val Leu Lys Ser Leu Asn Glu Glu Ala Val Lys 1165 1170 1175	3556
AAA GAC AAC TCT GTC CAT TGG GAG CGC CCT CAG AAA CCC AAG GCA CCA Lys Asp Asn Ser Val His Trp Glu Arg Pro Gln Lys Pro Lys Ala Pro 1180 1185 1190	3604
GTG GGG CAT TTT TAC GAA CCC CAG GCT CCC TCT GCT GAG GTG GAG ATG Val Gly His Phe Tyr Glu Pro Gln Ala Pro Ser Ala Glu Val Glu Met 1195 1200 1205	3652
ACA TCC TAT GTG CTC CTC GCT TAT CTC ACG GCC CAG CCA GCC CCA ACC Thr Ser Tyr Val Leu Leu Ala Tyr Leu Thr Ala Gln Pro Ala Pro Thr 1210 1215 1220	3700
TCG GAG GAC CTG ACC TCT GCA ACC AAC ATC GTG AAG TGG ATC ACG AAG Ser Glu Asp Leu Thr Ser Ala Thr Asn Ile Val Lys Trp Ile Thr Lys 1225 1230 1235 1240	3748
CAG CAG AAT GCC CAG GGC GGT TTC TCC TCC ACC CAG CAC ACA GTG GTG Gln Gln Asn Ala Gln Gly Gly Phe Ser Ser Thr Gln His Thr Val Val 1245 1250 1255	3796
GCT CTC CAT GCT CTG TCC AAA TAT GGA GCA GCC ACA TTT ACC AGG ACT Ala Leu His Ala Leu Ser Lys Tyr Gly Ala Ala Thr Phe Thr Arg Thr 1260 1265 1270	3844
GGG AAG GCT GCA CAG GTG ACT ATC CAG TCT TCA GGG ACA TTT TCC AGC Gly Lys Ala Ala Gln Val Thr Ile Gln Ser Ser Gly Thr Phe Ser Ser 1275 1280 1285	3892
AAA TTC CAA GTG GAC AAC AAC AAC CGC CTG TTA CTG CAG CAG GTC TCA Lys Phe Gln Val Asp Asn Asn Asn Arg Leu Leu Leu Gln Gln Val Ser 1290 1295 1300	3940
TTG CCA GAG CTG CCT GGG GAA TAC AGC ATG AAA GTG ACA GGA GAA GGA Leu Pro Glu Leu Pro Gly Glu Tyr Ser Met Lys Val Thr Gly Glu Gly 1305 1310 1315 1320	3988
TGT GTC TAC CTC CAG ACA TCC TTG AAA TAC AAT ATT CTC CCA GAA AAG Cys Val Tyr Leu Gln Thr Ser Leu Lys Tyr Asn Ile Leu Pro Glu Lys 1325 1330 1335	4036
GAA GAG TTC CCC TTT GCT TTA GGA GTG CAG ACT CTG CCT CAA ACT TGT Glu Glu Phe Pro Phe Ala Leu Gly Val Gln Thr Leu Pro Gln Thr Cys 1340 1345 1350	4084
GAT GAA CCC AAA GCC CAC ACC AGC TTC CAA ATC TCC CTA AGT GTC AGT Asp Glu Pro Lys Ala His Thr Ser Phe Gln Ile Ser Leu Ser Val Ser 1355 1360 1365	4132

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TAC ACA GGG AGC CGC TCT GCC TCC AAC ATG GCG ATC GTT GAT GTG AAG Tyr Thr Gly Ser Arg Ser Ala Ser Asn Met Ala Ile Val Asp Val Lys 1370 1375 1380	4180
ATG GTC TCT GGC TTC ATT CCC CTG AAG CCA ACA GTG AAA ATG CTT GAA Met Val Ser Gly Phe Ile Pro Leu Lys Pro Thr Val Lys Met Leu Glu 1385 1390 1395 1400	4228
AGA TCT AAC CAT GTG AGC CGG ACA GAA GTC AGC AGC AAC CAT GTC TTG Arg Ser Asn His Val Ser Arg Thr Glu Val Ser Ser Asn His Val Leu 1405 1410 1415	4276
ATT TAC CTT GAT AAG GTG TCA AAT CAG ACA CTG AGC TTG TTC TTC ACG Ile Tyr Leu Asp Lys Val Ser Asn Gln Thr Leu Ser Leu Phe Phe Thr 1420 1425 1430	4324
GTT CTG CAA GAT GTC CCA GTA AGA GAT CTC AAA CCA GCC ATA GTG AAA Val Leu Gln Asp Val Pro Val Arg Asp Leu Lys Pro Ala Ile Val Lys 1435 1440 1445	4372
GTC TAT GAT TAC TAC GAG ACG GAT GAG TTT GCA ATT GCT GAG TAC AAT Val Tyr Asp Tyr Tyr Glu Thr Asp Glu Phe Ala Ile Ala Glu Tyr Asn 1450 1455 1460	4420
GCT CCT TGC AGC AAA GAT CTT GGA AAT GCT TGAAGACCAC AAGGCTGAAA Ala Pro Cys Ser Lys Asp Leu Gly Asn Ala 1465 1470	4470
AGTGCTTTGC TGGAGTCCTG TTCTCTGAGC TCCACAGAAG ACACGTGTTT TTGTATCTTT	4530
AAAGACTTGA TGAATAAACA CTTTTTCTGG TCAAAAAAA	4569

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1474 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(E) FEATURES: bait region: 690-730

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Gly Lys Asn Lys Leu Leu His Pro Ser Leu Val Leu Leu Leu Leu 1 5 10 15	
Val Leu Leu Pro Thr Asp Ala Ser Val Ser Gly Lys Pro Gln Tyr Met 20 25 30	
Val Leu Val Pro Ser Leu Leu His Thr Glu Thr Thr Glu Lys Gly Cys 35 40 45	
Val Leu Leu Ser Tyr Leu Asn Glu Thr Val Thr Val Ser Ala Ser Leu 50 55 60	
Glu Ser Val Arg Gly Asn Arg Ser Leu Phe Thr Asp Leu Glu Ala Glu 65 70 75 80	

Asn Asp Val Leu His Cys Val Ala Phe Ala Val Pro Lys Ser Ser Ser  
 85 90 95  
 Asn Glu Glu Val Met Phe Leu Thr Val Gln Val Lys Gly Pro Thr Gln  
 100 105 110  
 Glu Phe Lys Lys Arg Thr Thr Val Met Val Lys Asn Glu Asp Ser Leu  
 115 120 125  
 Val Phe Val Gln Thr Asp Lys Ser Ile Tyr Lys Pro Gly Gln Thr Val  
 130 135 140  
 Lys Phe Arg Val Val Ser Met Asp Glu Asn Phe His Pro Leu Asn Glu  
 145 150 155 160  
 Leu Ile Pro Leu Val Tyr Ile Gln Asp Pro Lys Gly Asn Arg Ile Ala  
 165 170 175  
 Gln Trp Gln Ser Phe Gln Leu Glu Gly Gly Leu Lys Gln Phe Ser Phe  
 180 185 190  
 Pro Leu Ser Ser Glu Pro Phe Gln Gly Ser Tyr Lys Val Val Val Gln  
 195 200 205  
 Lys Lys Ser Gly Gly Arg Thr Glu His Pro Phe Thr Val Glu Glu Phe  
 210 215 220  
 Val Leu Pro Lys Phe Glu Val Gln Val Thr Val Pro Lys Ile Ile Thr  
 225 230 235 240  
 Ile Leu Glu Glu Glu Met Asn Val Ser Val Cys Gly Leu Tyr Thr Tyr  
 245 250 255  
 Gly Lys Pro Val Pro Gly His Val Thr Val Ser Ile Cys Arg Lys Tyr  
 260 265 270  
 Ser Asp Ala Ser Asp Cys His Gly Glu Asp Ser Gln Ala Phe Cys Glu  
 275 280 285  
 Lys Phe Ser Gly Gln Leu Asn Ser His Gly Cys Phe Tyr Gln Gln Val  
 290 295 300  
 Lys Thr Lys Val Phe Gln Leu Lys Arg Lys Glu Tyr Glu Met Lys Leu  
 305 310 315 320  
 His Thr Glu Ala Gln Ile Gln Glu Glu Gly Thr Val Val Glu Leu Thr  
 325 330 335  
 Gly Arg Gln Ser Ser Glu Ile Thr Arg Thr Ile Thr Lys Leu Ser Phe  
 340 345 350  
 Val Lys Val Asp Ser His Phe Arg Gln Gly Ile Pro Phe Phe Gly Gln  
 355 360 365  
 Val Arg Leu Val Asp Gly Lys Gly Val Pro Ile Pro Asn Lys Val Ile  
 370 375 380

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Phe Ile Arg Gly Asn Glu Ala Asn Tyr Tyr Ser Asn Ala Thr Thr Asp  
 385 390 395 400  
 Glu His Gly Leu Val Gln Phe Ser Ile Asn Thr Thr Asn Val Met Gly  
 405 410 415  
 Thr Ser Leu Thr Val Arg Val Asn Tyr Lys Asp Arg Ser Pro Cys Tyr  
 420 425 430  
 Gly Tyr Gln Trp Val Ser Glu Glu His Glu Glu Ala His His Thr Ala  
 435 440 445  
 Tyr Leu Val Phe Ser Pro Ser Lys Ser Phe Val His Leu Glu Pro Met  
 450 455 460  
 Ser His Glu Leu Pro Cys Gly His Thr Gln Thr Val Gln Ala His Tyr  
 465 470 475 480  
 Ile Leu Asn Gly Gly Thr Leu Leu Gly Leu Lys Lys Leu Ser Phe Tyr  
 485 490 495  
 Tyr Leu Ile Met Ala Lys Gly Gly Ile Val Arg Thr Gly Thr His Gly  
 500 505 510  
 Leu Leu Val Lys Gln Glu Asp Met Lys Gly His Phe Ser Ile Ser Ile  
 515 520 525  
 Pro Val Lys Ser Asp Ile Ala Pro Val Ala Arg Leu Leu Ile Tyr Ala  
 530 535 540  
 Val Leu Pro Thr Gly Asp Val Ile Gly Asp Ser Ala Lys Tyr Asp Val  
 545 550 555 560  
 Glu Asn Cys Leu Ala Asn Lys Val Asp Leu Ser Phe Ser Pro Ser Gln  
 565 570 575  
 Ser Leu Pro Ala Ser His Ala His Leu Arg Val Thr Ala Ala Pro Gln  
 580 585 590  
 Ser Val Cys Ala Leu Arg Ala Val Asp Gln Ser Val Leu Leu Met Lys  
 595 600 605  
 Pro Asp Ala Glu Leu Ser Ala Ser Ser Val Tyr Asn Leu Leu Pro Glu  
 610 615 620  
 Lys Asp Leu Thr Gly Phe Pro Gly Pro Leu Asn Asp Gln Asp Asp Glu  
 625 630 635 640  
 Asp Cys Ile Asn Arg His Asn Val Tyr Ile Asn Gly Ile Thr Tyr Thr  
 645 650 655  
 Pro Val Ser Ser Thr Asn Glu Lys Asp Met Tyr Ser Phe Leu Glu Asp  
 660 665 670  
 Met Gly Leu Lys Ala Phe Thr Asn Ser Lys Ile Arg Lys Pro Lys Met  
 675 680 685

Cys Pro Gln Leu Gln Gln Tyr Glu Met His Gly Pro Glu Gly Leu Arg  
 690 695 700  
 Val Gly Phe Tyr Glu Ser Asp Val Met Gly Arg Gly His Ala Arg Leu  
 705 710 715 720  
 Val His Val Glu Glu Pro His Thr Glu Thr Val Arg Lys Tyr Phe Pro  
 725 730 735  
 Glu Thr Trp Ile Trp Asp Leu Val Val Val Asn Ser Ala Gly Val Ala  
 740 745 750  
 Glu Val Gly Val Thr Val Pro Asp Thr Ile Thr Glu Trp Lys Ala Gly  
 755 760 765  
 Ala Phe Cys Leu Ser Glu Asp Ala Gly Leu Gly Ile Ser Ser Thr Ala  
 770 775 780  
 Ser Leu Arg Ala Phe Gln Pro Phe Phe Val Glu Leu Thr Met Pro Tyr  
 785 790 795 800  
 Ser Val Ile Arg Gly Glu Ala Phe Thr Leu Lys Ala Thr Val Leu Asn  
 805 810 815  
 Tyr Leu Pro Lys Cys Ile Arg Val Ser Val Gln Leu Glu Ala Ser Pro  
 820 825 830  
 Ala Phe Leu Ala Val Pro Val Glu Lys Glu Gln Ala Pro His Cys Ile  
 835 840 845  
 Cys Ala Asn Gly Arg Gln Thr Val Ser Trp Ala Val Thr Pro Lys Ser  
 850 855 860  
 Leu Gly Asn Val Asn Phe Thr Val Ser Ala Glu Ala Leu Glu Ser Gln  
 865 870 875 880  
 Glu Leu Cys Gly Thr Glu Val Pro Ser Val Pro Glu His Gly Arg Lys  
 885 890 895  
 Asp Thr Val Ile Lys Pro Leu Leu Val Glu Pro Glu Gly Leu Glu Lys  
 900 905 910  
 Glu Thr Thr Phe Asn Ser Leu Leu Cys Pro Ser Gly Gly Glu Val Ser  
 915 920 925  
 Glu Glu Leu Ser Leu Lys Leu Pro Pro Asn Val Val Glu Glu Ser Ala  
 930 935 940  
 Arg Ala Ser Val Ser Val Leu Gly Asp Ile Leu Gly Ser Ala Met Gln  
 945 950 955 960  
 Asn Thr Gln Asn Leu Leu Gln Met Pro Tyr Gly Cys Gly Glu Gln Asn  
 965 970 975  
 Met Val Leu Phe Ala Pro Asn Ile Tyr Val Leu Asp Tyr Leu Asn Glu  
 980 985 990

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Thr Gln Gln Leu Thr Pro Glu Ile Lys Ser Lys Ala Ile Gly Tyr Leu  
 995 1000 1005  
 Asn Thr Gly Tyr Gln Arg Gln Leu Asn Tyr Lys His Tyr Asp Gly Ser  
 1010 1015 1020  
 Tyr Ser Thr Phe Gly Glu Arg Tyr Gly Arg Asn Gln Gly Asn Thr Trp  
 1025 1030 1035 1040  
 Leu Thr Ala Phe Val Leu Lys Thr Phe Ala Gln Ala Arg Ala Tyr Ile  
 1045 1050 1055  
 Phe Ile Asp Glu Ala His Ile Thr Gln Ala Leu Ile Trp Leu Ser Gln  
 1060 1065 1070  
 Arg Gln Lys Asp Asn Gly Cys Phe Arg Ser Ser Gly Ser Leu Leu Asn  
 1075 1080 1085  
 Asn Ala Ile Lys Gly Gly Val Glu Asp Glu Val Thr Leu Ser Ala Tyr  
 1090 1095 1100  
 Ile Thr Ile Ala Leu Leu Glu Ile Pro Leu Thr Val Thr His Pro Val  
 1105 1110 1115 1120  
 Val Arg Asn Ala Leu Phe Cys Leu Glu Ser Ala Trp Lys Thr Ala Gln  
 1125 1130 1135  
 Glu Gly Asp His Gly Ser His Val Tyr Thr Lys Ala Leu Leu Ala Tyr  
 1140 1145 1150  
 Ala Phe Ala Leu Ala Gly Asn Gln Asp Lys Arg Lys Glu Val Leu Lys  
 1155 1160 1165  
 Ser Leu Asn Glu Glu Ala Val Lys Lys Asp Asn Ser Val His Trp Glu  
 1170 1175 1180  
 Arg Pro Gln Lys Pro Lys Ala Pro Val Gly His Phe Tyr Glu Pro Gln  
 1185 1190 1195 1200  
 Ala Pro Ser Ala Glu Val Glu Met Thr Ser Tyr Val Leu Leu Ala Tyr  
 1205 1210 1215  
 Leu Thr Ala Gln Pro Ala Pro Thr Ser Glu Asp Leu Thr Ser Ala Thr  
 1220 1225 1230  
 Asn Ile Val Lys Trp Ile Thr Lys Gln Gln Asn Ala Gln Gly Gly Phe  
 1235 1240 1245  
 Ser Ser Thr Gln His Thr Val Val Ala Leu His Ala Leu Ser Lys Tyr  
 1250 1255 1260  
 Gly Ala Ala Thr Phe Thr Arg Thr Gly Lys Ala Ala Gln Val Thr Ile  
 1265 1270 1275 1280  
 Gln Ser Ser Gly Thr Phe Ser Ser Lys Phe Gln Val Asp Asn Asn Asn  
 1285 1290 1295

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Arg Leu Leu Leu Gln Gln Val Ser Leu Pro Glu Leu Pro Gly Glu Tyr  
 1300 1305 1310  
 Ser Met Lys Val Thr Gly Glu Gly Cys Val Tyr Leu Gln Thr Ser Leu  
 1315 1320 1325  
 Lys Tyr Asn Ile Leu Pro Glu Lys Glu Glu Phe Pro Phe Ala Leu Gly  
 1330 1335 1340  
 Val Gln Thr Leu Pro Gln Thr Cys Asp Glu Pro Lys Ala His Thr Ser  
 1345 1350 1355 1360  
 Phe Gln Ile Ser Leu Ser Val Ser Tyr Thr Gly Ser Arg Ser Ala Ser  
 1365 1370 1375  
 Asn Met Ala Ile Val Asp Val Lys Met Val Ser Gly Phe Ile Pro Leu  
 1380 1385 1390  
 Lys Pro Thr Val Lys Met Leu Glu Arg Ser Asn His Val Ser Arg Thr  
 1395 1400 1405  
 Glu Val Ser Ser Asn His Val Leu Ile Tyr Leu Asp Lys Val Ser Asn  
 1410 1415 1420  
 Gln Thr Leu Ser Leu Phe Phe Thr Val Leu Gln Asp Val Pro Val Arg  
 1425 1430 1435 1440  
 Asp Leu Lys Pro Ala Ile Val Lys Val Tyr Asp Tyr Tyr Glu Thr Asp  
 1445 1450 1455  
 Glu Phe Ala Ile Ala Glu Tyr Asn Ala Pro Cys Ser Lys Asp Leu Gly  
 1460 1465 1470  
 Asn Ala

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 4599 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: Y

(iv) ANTI-SENSE: N

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 29..4480
- (D) OTHER INFORMATION:



## (ix) FEATURE:

(A) NAME/KEY: insertion\_seq

(B) LOCATION: 2102..2305

(D) OTHER INFORMATION:

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GTCTCCTCCA GCTCCTTCTT TCTGCAAC ATG GGG AAG AAC AAA CTC CTT CAT	52
Met Gly Lys Asn Lys Leu Leu His	
1 5	
CCA AGT CTG GTT CTT CTC CTC TTG GTC CTC CTG CCC ACA GAC GCC TCA	100
Pro Ser Leu Val Leu Leu Leu Val Leu Leu Pro Thr Asp Ala Ser	
10 15 20	
GTC TCT GGA AAA CCG CAG TAT ATG GTT CTG GTC CCC TCC CTG CTC CAC	148
Val Ser Gly Lys Pro Gln Tyr Met Val Leu Val Pro Ser Leu Leu His	
25 30 35 40	
ACT GAG ACC ACT GAG AAG GGC TGT GTC CTT CTG AGC TAC CTG AAT GAG	196
Thr Glu Thr Thr Glu Lys Gly Cys Val Leu Leu Ser Tyr Leu Asn Glu	
45 50 55	
ACA GTG ACT GTA AGT GCT TCC TTG GAG TCT GTC AGG GGA AAC AGG AGC	244
Thr Val Thr Val Ser Ala Ser Leu Glu Ser Val Arg Gly Asn Arg Ser	
60 65 70	
CTC TTC ACT GAC CTG GAG GCG GAG AAT GAC GTA CTC CAC TGT GTC GCC	292
Leu Phe Thr Asp Leu Glu Ala Glu Asn Asp Val Leu His Cys Val Ala	
75 80 85	
TTC GCT GTC CCA AAG TCT TCA TCC AAT GAG GAG GTA ATG TTC CTC ACT	340
Phe Ala Val Pro Lys Ser Ser Ser Asn Glu Glu Val Met Phe Leu Thr	
90 95 100	
GTC CAA GTG AAA GGA CCA ACC CAA GAA TTT AAG AAG CGG ACC ACA GTG	388
Val Gln Val Lys Gly Pro Thr Gln Glu Phe Lys Lys Arg Thr Thr Val	
105 110 115 120	
ATG GTT AAG AAC GAG GAC AGT CTG GTC TTT GTC CAG ACA GAC AAA TCA	436
Met Val Lys Asn Glu Asp Ser Leu Val Phe Val Gln Thr Asp Lys Ser	
125 130 135	
ATC TAC AAA CCA GGG CAG ACA GTG AAA TTT CGT GTT GTC TCC ATG GAT	484
Ile Tyr Lys Pro Gly Gln Thr Val Lys Phe Arg Val Val Ser Met Asp	
140 145 150	
GAA AAC TTT CAC CCC CTG AAT GAG TTG ATT CCA CTA GTA TAC ATT CAG	532
Glu Asn Phe His Pro Leu Asn Glu Leu Ile Pro Leu Val Tyr Ile Gln	
155 160 165	
GAT CCC AAA GGA AAT CGC ATC GCA CAA TGG CAG AGT TTC CAG TTA GAG	580
Asp Pro Lys Gly Asn Arg Ile Ala Gln Trp Gln Ser Phe Gln Leu Glu	
170 175 180	

48

GGT Gly 185	GGC Gly	CTC Leu	AAG Lys	CAA Gln	TTT Phe	TCT Ser	TTT Phe	CCC Pro	CTC Leu	TCA Ser	TCA Ser	GAG Glu	CCC Pro	TTC Phe	CAG Gln 200	628
GGC Gly	TCC Ser	TAC Tyr	AAG Lys	GTG Val 205	GTG Val	GTA Val	CAG Gln	AAG Lys	AAA Lys 210	TCA Ser	GGT Gly	GGA Gly	AGG Arg	ACA Thr 215	GAG Glu	676
CAC His	CCT Pro	TTC Phe	ACC Thr 220	GTG Val	GAG Glu	GAA Glu	TTT Phe	GTT Val 225	CTT Leu	CCC Pro	AAG Lys	TTT Phe	GAA Glu 230	GTA Val	CAA Gln	724
GTA Val	ACA Thr	GTG Val 235	CCA Pro	AAG Lys	ATA Ile	ATC Ile	ACC Thr 240	ATC Ile	TTG Leu	GAA Glu	GAA Glu	GAG Glu	ATG Met	AAT Asn	GTA Val	772
TCA Ser	GTG Val 250	TGT Cys	GGC Gly	CTA Leu	TAC Tyr	ACA Thr 255	TAT Tyr	GGG Gly	AAG Lys	CCT Pro	GTC Val 260	CCT Pro	GGA Gly	CAT His	GTG Val	820
ACT Thr 265	GTG Val	AGC Ser	ATT Ile	TGC Cys	AGA Arg 270	AAG Lys	TAT Tyr	AGT Ser	GAC Asp	GCT Ala 275	TCC Ser	GAC Asp	TGC Cys	CAC His	GGT Gly 280	868
GAA Glu	GAT Asp	TCA Ser	CAG Gln	GCT Ala 285	TTC Phe	TGT Cys	GAG Glu	AAA Lys	TTC Phe 290	AGT Ser	GGA Gly	CAG Gln	CTA Leu	AAC Asn 295	AGC Ser	916
CAT His	GGC Gly	TGC Cys	TTC Phe 300	TAT Tyr	CAG Gln	CAA Gln	GTA Val	AAA Lys 305	ACC Thr	AAG Lys	GTC Val	TTC Phe	CAG Gln 310	CTG Leu	AAG Lys	964
AGG Arg	AAG Lys	GAG Glu 315	TAT Tyr	GAA Glu	ATG Met	AAA Lys	CTT Leu 320	CAC His	ACT Thr	GAG Glu	GCC Ala	CAG Gln 325	ATC Ile	CAA Gln	GAA Glu	1012
GAA Glu	GGA Gly	ACA Thr	GTG Val 330	GTG Val	GAA Glu	TTG Leu 335	ACT Thr	GGA Gly	AGG Arg	CAG Gln	TCC Ser 340	AGT Ser	GAA Glu	ATC Ile	ACA Thr	1060
AGA Arg 345	ACC Thr	ATA Ile	ACC Thr	AAA Lys	CTC Leu 350	TCA Ser	TTT Phe	GTG Val	AAA Lys 355	GTG Val	GAC Asp	TCA Ser	CAC His	TTT Phe	CGA Arg 360	1108
CAG Gln	GGA Gly	ATT Ile	CCC Pro	TTC Phe 365	TTT Phe	GGG Gly	CAG Gln	GTG Val	CGC Arg 370	CTA Leu	GTA Val	GAT Asp	GGG Gly	AAA Lys 375	GGC Gly	1156
GTC Val	CCT Pro	ATA Ile	CCA Pro	AAT Asn 380	AAA Lys	GTC Val	ATA Ile	TTC Phe 385	ATC Ile	AGA Arg	GGA Gly	AAT Asn	GAA Glu 390	GCA Ala	AAC Asn	1204
TAT Tyr	TAC Tyr	TCC Ser	AAT Asn	GCT Ala 395	ACC Thr	ACG Thr	GAT Asp	GAG Glu 400	CAT His	GGC Gly	CTT Leu	GTA Val	CAG Gln	TTC Phe	TCT Ser	1252

ATC Ile 410	AAC Asn 410	ACC Thr 410	ACC Thr 410	AAT Asn 410	GTT Val 415	ATG Met 415	GGT Gly 415	ACC Thr 415	TCT Ser 415	CTT Leu 420	ACT Thr 420	GTT Val 420	AGG Arg 420	GTC Val 420	AAT Asn 420	1300
TAC Tyr 425	AAG Lys 425	GAT Asp 425	CGT Arg 425	AGT Ser 430	CCC Pro 430	TGT Cys 430	TAC Tyr 435	GGC Gly 435	TAC Tyr 435	CAG Gln 435	TGG Trp 435	GTG Val 440	TCA Ser 440	GAA Glu 440	GAA Glu 440	1348
CAC His 445	GAA Glu 445	GAG Glu 445	GCA Ala 445	CAT His 445	CAC His 445	ACT Thr 445	GCT Ala 450	TAT Tyr 450	CTT Leu 450	GTG Val 450	TTC Phe 455	TCC Ser 455	CCA Pro 455	AGC Ser 455	AAG Lys 455	1396
AGC Ser 460	TTT Phe 460	GTC Val 460	CAC His 460	CTT Leu 460	GAG Glu 460	CCC Pro 465	ATG Met 465	TCT Ser 465	CAT His 465	GAA Glu 465	CTA Leu 470	CCC Pro 470	TGT Cys 470	GGC Gly 470	CAT His 470	1444
ACT Thr 475	CAG Gln 475	ACA Thr 475	GTC Val 475	CAG Gln 475	GCA Ala 480	CAT His 480	TAT Tyr 480	ATT Ile 480	CTG Leu 480	AAT Asn 480	GGA Gly 485	GGC Gly 485	ACC Thr 485	CTG Leu 485	CTG Leu 485	1492
GGG Gly 490	CTG Leu 490	AAG Lys 490	AAG Lys 490	CTC Leu 495	TCC Ser 495	TTC Phe 495	TAT Tyr 495	TAT Tyr 495	CTG Leu 500	ATA Ile 500	ATG Met 500	GCA Ala 500	AAG Lys 500	GGA Gly 500	GGC Gly 500	1540
ATT Ile 505	GTC Val 505	CGA Arg 505	ACT Thr 510	GGG Gly 510	ACT Thr 510	CAT His 510	GGA Gly 515	CTG Leu 515	CTT Leu 515	GTG Val 515	AAG Lys 515	CAG Gln 515	GAA Glu 520	GAC Asp 520	ATG Met 520	1588
AAG Lys 525	GGC Gly 525	CAT His 525	TTT Phe 525	TCC Ser 525	ATC Ile 530	TCA Ser 530	ATC Ile 530	CCT Pro 530	GTG Val 530	AAG Lys 530	TCA Ser 535	GAC Asp 535	ATT Ile 535	GCT Ala 535	CCT Pro 535	1636
GTC Val 540	GCT Ala 540	CGG Arg 540	TTG Leu 540	CTC Leu 540	ATC Ile 545	TAT Tyr 545	GCT Ala 545	GTT Val 545	TTA Leu 545	CCT Pro 550	ACC Thr 550	GGG Gly 550	GAC Asp 550	GTG Val 550	ATT Ile 550	1684
GGG Gly 555	GAT Asp 555	TCT Ser 555	GCA Ala 560	AAA Lys 560	TAT Tyr 560	GAT Asp 560	GTT Val 560	GAA Glu 560	AAT Asn 565	TGT Cys 565	CTG Leu 565	GCC Ala 565	AAC Asn 565	AAG Lys 565	GTG Val 565	1732
GAT Asp 570	TTG Leu 570	AGC Ser 570	TTC Phe 575	AGC Ser 575	CCA Pro 575	TCA Ser 575	CAA Gln 580	AGT Ser 580	CTC Leu 580	CCA Pro 580	GCC Ala 580	TCA Ser 580	CAC His 580	GCC Ala 580	CAC His 580	1780
CTG Leu 585	CGA Arg 590	GTC Val 590	ACA Thr 590	GCG Ala 590	GCT Ala 590	CCT Pro 595	CAG Gln 595	TCC Ser 595	GTC Val 595	TGC Cys 595	GCC Ala 595	CTC Leu 600	CGT Arg 600	GCT Ala 600	GTG Val 600	1828
GAC Asp 605	CAA Gln 605	AGC Ser 605	GTG Val 605	CTG Leu 605	CTC Leu 605	ATG Met 610	AAG Lys 610	CCT Pro 610	GAT Asp 610	GCT Ala 615	GAG Glu 615	CTC Leu 615	TCG Ser 615	GCG Ala 615	TCC Ser 615	1876
TCG Ser 620	GTT Val 620	TAC Tyr 620	AAC Asn 620	CTG Leu 620	CTA Leu 625	CCA Pro 625	GAA Glu 625	AAG Lys 625	GAC Asp 625	CTC Leu 630	ACT Thr 630	GGC Gly 630	TTC Phe 630	CCT Pro 630	GGG Gly 630	1924

50

CCT TTG AAT GAC CAG GAC GAT GAA GAC TGC ATC AAT CGT CAT AAT GTC Pro Leu Asn Asp Gln Asp Asp Glu Asp Cys Ile Asn Arg His Asn Val 635 640 645	1972
TAT ATT AAT GGA ATC ACA TAT ACT CCA GTA TCA AGT ACA AAT GAA AAG Tyr Ile Asn Gly Ile Thr Tyr Thr Pro Val Ser Thr Asn Glu Lys 650 655 660	2020
GAT ATG TAC AGC TTC CTA GAG GAC ATG GGC TTA AAG GCA TTC ACC AAC Asp Met Tyr Ser Phe Leu Glu Asp Met Gly Leu Lys Ala Phe Thr Asn 665 670 675 680	2068
TCA AAG ATT CGT AAA CCC AAA ATG TGT CCA CAG CTG CAG TCA GTG TCA Ser Lys Ile Arg Lys Pro Lys Met Cys Pro Gln Leu Gln Ser Val Ser 685 690 695	2116
GCC GGC GCC GTG GGA CAG GGA TAT TAT GGA GCC GGA CTG GGA GTG GTG Ala Gly Ala Val Gly Gln Gly Tyr Tyr Gly Ala Gly Leu Gly Val Val 700 705 710	2164
GAG AGG CCT TAT GTG CCT CAG CTG GGT ACC TAT AAT GTG ATC CCT CTG Glu Arg Pro Tyr Val Pro Gln Leu Gly Thr Tyr Asn Val Ile Pro Leu 715 720 725	2212
AAT AAT GAG CAG AGC TCA GGA CCT GTG CCT GAG ACA GTG AGG AAG TAT Asn Asn Glu Gln Ser Ser Gly Pro Val Pro Glu Thr Val Arg Lys Tyr 730 735 740	2260
TTC CCT GAG ACA TGG ATC TGG GAT CTG GTG GTG GTG AAT TCC GCG GGT Phe Pro Glu Thr Trp Ile Trp Asp Leu Val Val Val Asn Ser Ala Gly 745 750 755 760	2308
GTG GCT GAG GTA GGA GTA ACA GTC CCT GAC ACC ATC ACC GAG TGG AAG Val Ala Glu Val Gly Val Thr Val Pro Asp Thr Ile Thr Glu Trp Lys 765 770 775	2356
GCA GGG GCC TTC TGC CTG TCT GAA GAT GCT GGA CTT GGT ATC TCT TCC Ala Gly Ala Phe Cys Leu Ser Glu Asp Ala Gly Leu Gly Ile Ser Ser 780 785 790	2404
ACT GCC TCT CTC CGA GCC TTC CAG CCC TTC TTT GTG GAG CTC ACA ATG Thr Ala Ser Leu Arg Ala Phe Gln Pro Phe Phe Val Glu Leu Thr Met 795 800 805	2452
CCT TAC TCT GTG ATT CGT GGA GAG GCC TTC ACA CTC AAG GCC ACG GTC Pro Tyr Ser Val Ile Arg Gly Glu Ala Phe Thr Leu Lys Ala Thr Val 810 815 820	2500
CTA AAC TAC CTT CCC AAA TGC ATC CGG GTC AGT GTG CAG CTG GAA GCC Leu Asn Tyr Leu Pro Lys Cys Ile Arg Val Ser Val Gln Leu Glu Ala 825 830 835 840	2548
TCT CCC GCC TTC CTA GCT GTC CCA GTG GAG AAG GAA CAA GCG CCT CAC Ser Pro Ala Phe Leu Ala Val Pro Val Glu Lys Glu Gln Ala Pro His 845 850 855	2596

51

TGC Cys	ATC Ile	TGT Cys	GCA Ala 860	AAC Asn	GGG Gly	CGG Arg	CAA Gln 865	ACT Thr	GTG Val	TCC Ser	TGG Trp	GCA Ala 870	GTA Val	ACC Thr	CCA Pro	2644
AAG Lys	TCA Ser	TTA Leu 875	GGA Gly	AAT Asn	GTG Val	AAT Asn	TTC Phe 880	ACT Thr	GTG Val	AGC Ser	GCA Ala	GAG Glu 885	GCA Ala	CTA Leu	GAG Glu	2692
TCT Ser	CAA Gln 890	GAG Glu	CTG Leu	TGT Cys	GGG Gly	ACT Thr 895	GAG Glu	GTG Val	CCT Pro	TCA Ser	GTT Val 900	CCT Pro	GAA Glu	CAC His	GGA Gly	2740
AGG Arg 905	AAA Lys	GAC Asp	ACA Thr	GTC Val	ATC Ile 910	AAG Lys	CCT Pro	CTG Leu	TTG Leu	GTT Val 915	GAA Glu	CCT Pro	GAA Glu	GGA Gly	CTA Leu 920	2788
GAG Glu	AAG Lys	GAA Glu	ACA Thr 925	ACA Thr	TTC Phe	AAC Asn	TCC Ser	CTA Leu	CTT Leu 930	TGT Cys	CCA Pro	TCA Ser	GGT Gly 935	GGT Gly	GAG Glu	2836
GTT Val	TCT Ser	GAA Glu 940	GAA Glu	TTA Leu	TCC Ser	CTG Leu	AAA Lys	CTG Leu 945	CCA Pro	CCA Pro	AAT Asn	GTG Val 950	GTA Val	GAA Glu	GAA Glu	2884
TCT Ser	GCC Ala 955	CGA Arg	GCT Ala	TCT Ser	GTC Val	TCA Ser	GTT Val 960	TTG Leu	GGA Gly	GAC Asp	ATA Ile	TTA Leu 965	GGC Gly	TCT Ser	GCC Ala	2932
ATG Met 970	CAA Gln	AAC Asn	ACA Thr	CAA Gln	AAT Asn	CTT Leu 975	CTC Leu	CAG Gln	ATG Met	CCC Pro	TAT Tyr 980	GGC Gly	TGT Cys	GGA Gly	GAG Glu	2980
CAG Gln 985	AAT Asn	ATG Met	GTC Val	CTC Leu	TTT Phe 990	GCT Ala	CCT Pro	AAC Asn	ATC Ile	TAT Tyr 995	GTA Val	CTG Leu	GAT Asp	TAT Tyr	CTA Leu 1000	3028
AAT Asn	GAA Glu	ACA Thr	CAG Gln 1005	CAG Gln	CTT Leu	ACT Thr	CCA Pro	GAG Glu	ATC Ile 1010	AAG Lys	TCC Ser	AAG Lys	GCC Ala 1015	ATT Ile	GGC Gly	3076
TAT Tyr	CTC Leu	AAC Asn	ACT Thr 1020	GGT Gly	TAC Tyr	CAG Gln	AGA Arg	CAG Gln	TTG Leu	AAC Asn	TAC Tyr	AAA Lys	CAC His	TAT Tyr	GAT Asp	3124
GGC Gly	TCC Ser	TAC Tyr 1035	AGC Ser	ACC Thr	TTT Phe	GGG Gly	GAG Glu 1040	CGA Arg	TAT Tyr	GGC Gly	AGG Arg	AAC Asn 1045	CAG Gln	GGC Gly	AAC Asn	3172
ACC Thr 1050	TGG Trp	CTC Leu	ACA Thr	GCC Ala	TTT Phe	GTT Val 1055	CTG Leu	AAG Lys	ACT Thr	TTT Phe	GCC Ala 1060	CAA Gln	GCT Ala	CGA Arg	GCC Ala	3220
TAC Tyr 1065	ATC Ile	TTC Phe	ATC Ile	GAT Asp	GAA Glu 1070	GCA Ala	CAC His	ATT Ile	ACC Thr	CAA Gln 1075	GCC Ala	CTC Leu	ATA Ile	TGG Trp	CTC Leu 1080	3268

TCC CAG AGG CAG AAG GAC AAT GGC TGT TTC AGG AGC TCT GGG TCA CTG Ser Gln Arg Gln Lys Asp Asn Gly Cys Phe Arg Ser Ser Gly Ser Leu 1085 1090 1095	3316
CTC AAC AAT GCC ATA AAG GGA GGA GTA GAA GAT GAA GTG ACC CTC TCC Leu Asn Asn Ala Ile Lys Gly Gly Val Glu Asp Glu Val Thr Leu Ser 1100 1105 1110	3364
GCC TAT ATC ACC ATC GCC CTT CTG GAG ATT CCT CTC ACA GTC ACT CAC Ala Tyr Ile Thr Ile Ala Leu Leu Glu Ile Pro Leu Thr Val Thr His 1115 1120 1125	3412
CCT GTT GTC CGC AAT GCC CTG TTT TGC CTG GAG TCA GCC TGG AAG ACA Pro Val Val Arg Asn Ala Leu Phe Cys Leu Glu Ser Ala Trp Lys Thr 1130 1135 1140	3460
GCA CAA GAA GGG GAC CAT GGC AGC CAT GTA TAT ACC AAA GCA CTG CTG Ala Gln Glu Gly Asp His Gly Ser His Val Tyr Thr Lys Ala Leu Leu 1145 1150 1155 1160	3508
GCC TAT GCT TTT GCC CTG GCA GGT AAC CAG GAC AAG AGG AAG GAA GTA Ala Tyr Ala Phe Ala Leu Ala Gly Asn Gln Asp Lys Arg Lys Glu Val 1165 1170 1175	3556
CTC AAG TCA CTT AAT GAG GAA GCT GTG AAG AAA GAC AAC TCT GTC CAT Leu Lys Ser Leu Asn Glu Glu Ala Val Lys Lys Asp Asn Ser Val His 1180 1185 1190	3604
TGG GAG CGC CCT CAG AAA CCC AAG GCA CCA GTG GGG CAT TTT TAC GAA Trp Glu Arg Pro Gln Lys Pro Lys Ala Pro Val Gly His Phe Tyr Glu 1195 1200 1205	3652
CCC CAG GCT CCC TCT GCT GAG GTG GAG ATG ACA TCC TAT GTG CTC CTC Pro Gln Ala Pro Ser Ala Glu Val Glu Met Thr Ser Tyr Val Leu Leu 1210 1215 1220	3700
GCT TAT CTC ACG GCC CAG CCA GCC CCA ACC TCG GAG GAC CTG ACC TCT Ala Tyr Leu Thr Ala Gln Pro Ala Pro Thr Ser Glu Asp Leu Thr Ser 1225 1230 1235 1240	3748
GCA ACC AAC ATC GTG AAG TGG ATC ACG AAG CAG CAG AAT GCC CAG GGC Ala Thr Asn Ile Val Lys Trp Ile Thr Lys Gln Gln Asn Ala Gln Gly 1245 1250 1255	3796
GGT TTC TCC TCC ACC CAG CAC ACA GTG GTG GCT CTC CAT GCT CTG TCC Gly Phe Ser Ser Thr Gln His Thr Val Val Ala Leu His Ala Leu Ser 1260 1265 1270	3844
AAA TAT GGA GCA GCC ACA TTT ACC AGG ACT GGG AAG GCT GCA CAG GTG Lys Tyr Gly Ala Ala Thr Phe Thr Arg Thr Gly Lys Ala Ala Gln Val 1275 1280 1285	3892
ACT ATC CAG TCT TCA GGG ACA TTT TCC AGC AAA TTC CAA GTG GAC AAC Thr Ile Gln Ser Ser Gly Thr Phe Ser Ser Lys Phe Gln Val Asp Asn 1290 1295 1300	3940

53

AAC AAC CGC CTG TTA CTG CAG CAG GTC TCA TTG CCA GAG CTG CCT GGG	3988
Asn Asn Arg Leu Leu Leu Gln Gln Val Ser Leu Pro Glu Leu Pro Gly	
1305 1310 1315 1320	
GAA TAC AGC ATG AAA GTG ACA GGA GAA GGA TGT GTC TAC CTC CAG ACA	4036
Glu Tyr Ser Met Lys Val Thr Gly Glu Gly Cys Val Tyr Leu Gln Thr	
1325 1330 1335	
TCC TTG AAA TAC AAT ATT CTC CCA GAA AAG GAA GAG TTC CCC TTT GCT	4084
Ser Leu Lys Tyr Asn Ile Leu Pro Glu Lys Glu Glu Phe Pro Phe Ala	
1340 1345 1350	
TTA GGA GTG CAG ACT CTG CCT CAA ACT TGT GAT GAA CCC AAA GCC CAC	4132
Leu Gly Val Gln Thr Leu Pro Gln Thr Cys Asp Glu Pro Lys Ala His	
1355 1360 1365	
ACC AGC TTC CAA ATC TCC CTA AGT GTC AGT TAC ACA GGG AGC CGC TCT	4180
Thr Ser Phe Gln Ile Ser Leu Ser Val Ser Tyr Thr Gly Ser Arg Ser	
1370 1375 1380	
GCC TCC AAC ATG GCG ATC GTT GAT GTG AAG ATG GTC TCT GGC TTC ATT	4228
Ala Ser Asn Met Ala Ile Val Asp Val Lys Met Val Ser Gly Phe Ile	
1385 1390 1395 1400	
CCC CTG AAG CCA ACA GTG AAA ATG CTT GAA AGA TCT AAC CAT GTG AGC	4276
Pro Leu Lys Pro Thr Val Lys Met Leu Glu Arg Ser Asn His Val Ser	
1405 1410 1415	
CGG ACA GAA GTC AGC AGC AAC CAT GTC TTG ATT TAC CTT GAT AAG GTG	4324
Arg Thr Glu Val Ser Ser Asn His Val Leu Ile Tyr Leu Asp Lys Val	
1420 1425 1430	
TCA AAT CAG ACA CTG AGC TTG TTC TTC ACG GTT CTG CAA GAT GTC CCA	4372
Ser Asn Gln Thr Leu Ser Leu Phe Phe Thr Val Leu Gln Asp Val Pro	
1435 1440 1445	
GTA AGA GAT CTG AAA CCA GCC ATA GTG AAA GTC TAT GAT TAC TAC GAG	4420
Val Arg Asp Leu Lys Pro Ala Ile Val Lys Val Tyr Asp Tyr Tyr Glu	
1450 1455 1460	
ACG GAT GAG TTT GCA ATT GCT GAG TAC AAT GCT CCT TGC AGC AAA GAT	4468
Thr Asp Glu Phe Ala Ile Ala Glu Tyr Asn Ala Pro Cys Ser Lys Asp	
1465 1470 1475 1480	
CTT GGA AAT GCT TGAAGACCAC AAGGCTGAAA AGTGCTTTGC TGGAGTCCTG	4520
Leu Gly Asn Ala	
TTCTCTGAGC TCCACAGAAG ACACGTGTTT TTGTATCTTT AAAGACTTGA TGAATAAACA	4580
CTTTTCTGG TCAAAAAAA	4599

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1484 amino acids

(B) TYPE: amino acid

54

(D) TOPOLOGY: linear

(E) FEATURES: bait region: 690-740

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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Met Gly Lys Asn Lys Leu Leu His Pro Ser Leu Val Leu Leu Leu Leu
 1           5           10           15
Val Leu Leu Pro Thr Asp Ala Ser Val Ser Gly Lys Pro Gln Tyr Met
          20           25           30
Val Leu Val Pro Ser Leu Leu His Thr Glu Thr Thr Glu Lys Gly Cys
          35           40           45
Val Leu Leu Ser Tyr Leu Asn Glu Thr Val Thr Val Ser Ala Ser Leu
          50           55           60
Glu Ser Val Arg Gly Asn Arg Ser Leu Phe Thr Asp Leu Glu Ala Glu
          65           70           75           80
Asn Asp Val Leu His Cys Val Ala Phe Ala Val Pro Lys Ser Ser Ser
          85           90           95
Asn Glu Glu Val Met Phe Leu Thr Val Gln Val Lys Gly Pro Thr Gln
          100          105          110
Glu Phe Lys Lys Arg Thr Thr Val Met Val Lys Asn Glu Asp Ser Leu
          115          120          125
Val Phe Val Gln Thr Asp Lys Ser Ile Tyr Lys Pro Gly Gln Thr Val
          130          135          140
Lys Phe Arg Val Val Ser Met Asp Glu Asn Phe His Pro Leu Asn Glu
          145          150          155          160
Leu Ile Pro Leu Val Tyr Ile Gln Asp Pro Lys Gly Asn Arg Ile Ala
          165          170          175
Gln Trp Gln Ser Phe Gln Leu Glu Gly Gly Leu Lys Gln Phe Ser Phe
          180          185          190
Pro Leu Ser Ser Glu Pro Phe Gln Gly Ser Tyr Lys Val Val Val Gln
          195          200          205
Lys Lys Ser Gly Gly Arg Thr Glu His Pro Phe Thr Val Glu Glu Phe
          210          215          220
Val Leu Pro Lys Phe Glu Val Gln Val Thr Val Pro Lys Ile Ile Thr
          225          230          235          240
Ile Leu Glu Glu Glu Met Asn Val Ser Val Cys Gly Leu Tyr Thr Tyr
          245          250          255
Gly Lys Pro Val Pro Gly His Val Thr Val Ser Ile Cys Arg Lys Tyr
          260          265          270

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55

Ser Asp Ala Ser Asp Cys His Gly Glu Asp Ser Gln Ala Phe Cys Glu  
 275 280 285  
 Lys Phe Ser Gly Gln Leu Asn Ser His Gly Cys Phe Tyr Gln Gln Val  
 290 295 300  
 Lys Thr Lys Val Phe Gln Leu Lys Arg Lys Glu Tyr Glu Met Lys Leu  
 305 310 315 320  
 His Thr Glu Ala Gln Ile Gln Glu Glu Gly Thr Val Val Glu Leu Thr  
 325 330 335  
 Gly Arg Gln Ser Ser Glu Ile Thr Arg Thr Ile Thr Lys Leu Ser Phe  
 340 345 350  
 Val Lys Val Asp Ser His Phe Arg Gln Gly Ile Pro Phe Phe Gly Gln  
 355 360 365  
 Val Arg Leu Val Asp Gly Lys Gly Val Pro Ile Pro Asn Lys Val Ile  
 370 375 380  
 Phe Ile Arg Gly Asn Glu Ala Asn Tyr Tyr Ser Asn Ala Thr Thr Asp  
 385 390 395 400  
 Glu His Gly Leu Val Gln Phe Ser Ile Asn Thr Thr Asn Val Met Gly  
 405 410 415  
 Thr Ser Leu Thr Val Arg Val Asn Tyr Lys Asp Arg Ser Pro Cys Tyr  
 420 425 430  
 Gly Tyr Gln Trp Val Ser Glu Glu His Glu Glu Ala His His Thr Ala  
 435 440 445  
 Tyr Leu Val Phe Ser Pro Ser Lys Ser Phe Val His Leu Glu Pro Met  
 450 455 460  
 Ser His Glu Leu Pro Cys Gly His Thr Gln Thr Val Gln Ala His Tyr  
 465 470 475 480  
 Ile Leu Asn Gly Gly Thr Leu Leu Gly Leu Lys Lys Leu Ser Phe Tyr  
 485 490 495  
 Tyr Leu Ile Met Ala Lys Gly Gly Ile Val Arg Thr Gly Thr His Gly  
 500 505 510  
 Leu Leu Val Lys Gln Glu Asp Met Lys Gly His Phe Ser Ile Ser Ile  
 515 520 525  
 Pro Val Lys Ser Asp Ile Ala Pro Val Ala Arg Leu Leu Ile Tyr Ala  
 530 535 540  
 Val Leu Pro Thr Gly Asp Val Ile Gly Asp Ser Ala Lys Tyr Asp Val  
 545 550 555 560  
 Glu Asn Cys Leu Ala Asn Lys Val Asp Leu Ser Phe Ser Pro Ser Gln  
 565 570 575

56

Ser Leu Pro Ala Ser His Ala His Leu Arg Val Thr Ala Ala Pro Gln  
 580 585 590  
 Ser Val Cys Ala Leu Arg Ala Val Asp Gln Ser Val Leu Leu Met Lys  
 595 600 605  
 Pro Asp Ala Glu Leu Ser Ala Ser Ser Val Tyr Asn Leu Leu Pro Glu  
 610 615 620  
 Lys Asp Leu Thr Gly Phe Pro Gly Pro Leu Asn Asp Gln Asp Asp Glu  
 625 630 635 640  
 Asp Cys Ile Asn Arg His Asn Val Tyr Ile Asn Gly Ile Thr Tyr Thr  
 645 650 655  
 Pro Val Ser Ser Thr Asn Glu Lys Asp Met Tyr Ser Phe Leu Glu Asp  
 660 665 670  
 Met Gly Leu Lys Ala Phe Thr Asn Ser Lys Ile Arg Lys Pro Lys Met  
 675 680 685  
 Cys Pro Gln Leu Gln Ser Val Ser Ala Gly Ala Val Gly Gln Gly Tyr  
 690 695 700  
 Tyr Gly Ala Gly Leu Gly Val Val Glu Arg Pro Tyr Val Pro Gln Leu  
 705 710 715 720  
 Gly Thr Tyr Asn Val Ile Pro Leu Asn Asn Glu Gln Ser Ser Gly Pro  
 725 730 735  
 Val Pro Glu Thr Val Arg Lys Tyr Phe Pro Glu Thr Trp Ile Trp Asp  
 740 745 750  
 Leu Val Val Val Asn Ser Ala Gly Val Ala Glu Val Gly Val Thr Val  
 755 760 765  
 Pro Asp Thr Ile Thr Glu Trp Lys Ala Gly Ala Phe Cys Leu Ser Glu  
 770 775 780  
 Asp Ala Gly Leu Gly Ile Ser Ser Thr Ala Ser Leu Arg Ala Phe Gln  
 785 790 795 800  
 Pro Phe Phe Val Glu Leu Thr Met Pro Tyr Ser Val Ile Arg Gly Glu  
 805 810 815  
 Ala Phe Thr Leu Lys Ala Thr Val Leu Asn Tyr Leu Pro Lys Cys Ile  
 820 825 830  
 Arg Val Ser Val Gln Leu Glu Ala Ser Pro Ala Phe Leu Ala Val Pro  
 835 840 845  
 Val Glu Lys Glu Gln Ala Pro His Cys Ile Cys Ala Asn Gly Arg Gln  
 850 855 860  
 Thr Val Ser Trp Ala Val Thr Pro Lys Ser Leu Gly Asn Val Asn Phe  
 865 870 875 880

57

Thr Val Ser Ala Glu Ala Leu Glu Ser Gln Glu Leu Cys Gly Thr Glu  
 885 890 895  
 Val Pro Ser Val Pro Glu His Gly Arg Lys Asp Thr Val Ile Lys Pro  
 900 905 910  
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 His Val Tyr Thr Lys Ala Leu Leu Ala Tyr Ala Phe Ala Leu Ala Gly  
 1155 1160 1165  
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 1170 1175 1180

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Val Lys Lys Asp Asn Ser Val His Trp Glu Arg Pro Gln Lys Pro Lys  
 1185 1190 1195 1200  
 Ala Pro Val Gly His Phe Tyr Glu Pro Gln Ala Pro Ser Ala Glu Val  
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 Val Lys Val Tyr Asp Tyr Tyr Glu Thr Asp Glu Phe Ala Ile Ala Glu  
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 Tyr Asn Ala Pro Cys Ser Lys Asp Leu Gly Asn Ala  
 1475 1480

**PATENT CLAIMS**

1. A process for the production of recombinant  $\alpha$ -macroglobulin, variants, fragments or derivatives thereof, wherein a functionally operative expression vector comprising a gene encoding for the expression of  $\alpha$ -  
5 macroglobulin, variants, fragments or derivatives thereof, or alleles of such a gene, is introduced into a suitable host capable of expressing said gene, said host is cultured in a suitable nutrient medium containing sources of assimilable carbon and nitrogen and other essential nutrients, and the expressed  $\alpha$ -macroglobulin or fragments or derivatives thereof is recovered.  
10
2. The process of claim 1, wherein said gene encodes for the expression of human  $\alpha_2$ -macroglobulin, variants, fragments or derivatives thereof.
- 15 3. The process of claim 2, wherein said gene encodes for the expression of human  $\alpha_2$ -macroglobulin having the amino acid sequence of SEQ ID NO:2, or a fragment or derivative thereof.
4. The process of claim 2 or 3, wherein said gene comprises the DNA  
20 sequence of SEQ ID NO:1, or a fragment thereof.
5. The process of claim 1 or 2, wherein said gene encodes for a variant  $\alpha$ -macroglobulin, in which the amino acid sequence of the bait region has been altered.  
25
6. The process of claim 5, wherein the bait region has been altered by incorporation of further proteinase target sites.
7. The process of claim 5, wherein the bait region has been altered  
30 by removal of proteinase target sites.
8. The process of claim 5, wherein the bait region has been altered by replacing one or more specific proteinase target sites with one or more other specific proteinase target sites.  
35
9. The process of claim 8, wherein said proteinase target sites are specific for bovine trypsin, Streptomyces griseus trypsin, papain, porcine elastase, bovine chymosin, bovine chymotrypsin, Staphylococcus aureus strain

V8 proteinase, human plasmin, bovine thrombin, thermolysin, subtilisin Novo and/or Streptomyces griseus proteinase B.

10. The process of claim 5, wherein wherein the bait region has been  
5 altered by replacing said bait region or part thereof with a bait region or a part thereof from another  $\alpha$ -macroglobulin.

11. The process of claim 10, wherein said bait regions originate from  
human  $\alpha_2$ M, Pregnancy Zone Protein (PZP), rat  $\alpha_1$ M, rat  $\alpha_2$ M, rat  $\alpha_1$ I<sub>3</sub> variant  
10 1, or rat  $\alpha_1$ I<sub>3</sub> variant 2 ( $\alpha_1$ I<sub>3</sub> =  $\alpha_1$ -inhibitor 3), especially PZP.

12. The process of any of claims 5 to 11, wherein said gene encodes  
for the expression of human  $\alpha_2$ -macroglobulin variant having the amino acid  
sequence of SEQ ID NO:4, or a fragment or derivative thereof.

15 13. The process of any of claims 5 to 12, wherein said gene comprises  
the DNA sequence of SEQ ID NO:3, or a fragment thereof.

14. The process of any of the claims 1 to 13, wherein said gene is  
20 a synthetic gene.

15. The process of any of the claims 1 to 14, wherein said  $\alpha$ -  
macroglobulin, variant, fragment or derivative thereof is co-expressed with  
a desired gene product.

25 16. The process of any of the claims 1 to 15, wherein said gene is,  
or is derived from, a human gene.

17. The process of any of the claims 1 to 16, wherein said host is  
30 a bacterial strain, a fungal strain, a mammalian cell line, or a mammal.

18. The process of claim 17, wherein said host is a fungus.

19. The process of claim 18, wherein said fungus belongs to the genus  
35 Aspergillus.

20 The process of claim 18, wherein said host is a yeast.

21. The process of claim 20, wherein said yeast belongs to the genus Saccharomyces.
22. The process of claim 17, wherein said host is a mammalian cell  
5 line.
23. The process of claim 22, wherein said mammalian cell line is a Syrian Baby Hamster Kidney (BKH) cell line.
- 10 24. The process of claim 23, wherein said cell line is available from ATCC under No. CRL 1632.
25. A DNA sequence comprising a gene encoding for the expression of an  $\alpha$ -macroglobulin, variants, fragments or derivatives thereof.
- 15 26 The DNA sequence of claim 25, wherein said gene encodes for human  $\alpha_2$ -macroglobulin.
27. The DNA sequence of claim 25, wherein said gene encodes for the amino  
20 acid sequence of SEQ ID NO:2 or a fragment or derivative thereof.
28. The DNA sequence of claim 26 or 27, wherein said gene has the nucleotide sequence of SEQ ID NO:1 or a fragment thereof.
- 25 29. The DNA sequence of claim 25 or 26, wherein said gene encodes for a variant  $\alpha$ -macroglobulin, in which the amino acid sequence of the bait region has been altered.
30. The DNA sequence of claim 29, wherein said bait region has been  
30 altered by incorporation of further proteinase target sites.
31. The DNA sequence of claim 29, wherein said bait region has been altered by removal of proteinase target sites.
- 35 32. The DNA sequence of claim 29, wherein said bait region has been altered by replacing one or more specific proteinase target sites with one or more other specific proteinase target sites.

33. The DNA sequence of claim 29, wherein, wherein said proteinase target sites are specific for bovine trypsin, Streptomyces griseus trypsin, papain, porcine elastase, bovine chymosin, bovine chymotrypsin, Staphylococcus aureus strain V8 proteinase, human plasmin, bovine thrombin, thermolysin, subtilisin Novo and/or Streptomyces griseus proteinase B.
34. The DNA sequence of claim 29, wherein the bait region has been altered by replacing said bait region or part thereof with a bait region or a part thereof from another  $\alpha$ -macroglobulin.
35. The DNA sequence of claim 34, wherein said bait region originates from human  $\alpha_2$ M, Pregnancy Zone Protein (PZP), rat  $\alpha_1$ M, rat  $\alpha_2$ M, rat  $\alpha_1$ I<sub>3</sub> variant 1, or rat  $\alpha_1$ I<sub>3</sub> variant 2, especially PZP.
36. A functionally operative expression vector comprising a gene in accordance with any of the claims 25 to 35 for the expression of human  $\alpha_2$ -macroglobulin, variants, fragments or derivatives thereof, or alleles of such a gene.
37. The vector of claim 36, further comprising regulatory elements necessary for the stable maintenance of said vector in mammalian cells.
38. The vector of claim 36 or 37, further comprising sequences providing for the processing and secretion of the expressed product.
39. The vector of any of the claims 36 to 38, further comprising one or more other genes encoding for a desired gene product.
40. A functionally operative expression vector comprising a gene encoding for the expression of an  $\alpha$ -macroglobulin, variants, fragments or derivatives thereof, or alleles of such a gene, essentially as described.
41. A transformed host comprising a functionally operative expression vector comprising a gene encoding for the expression of human  $\alpha_2$ -macroglobulin or fragments or derivatives thereof, or alleles of such a gene.
42. The host of claim 41, wherein said vector is the vector of any of the claims 36 to 40.



43. The host of claim 41 or 42, wherein said host is a bacterial strain, a fungal strain, a mammalian cell line, or a mammal.
44. The host of claim 43, wherein said host is a fungus.
45. The host of claim 44, wherein said fungus belongs to the genus Aspergillus.
46. The host of claim 44, wherein said host is a yeast.
47. The host of claim 46, wherein said host belongs to the genus Saccharomyces.
48. The host of claim 43, wherein said host is a mammalian cell line.
49. The host of claim 48, wherein said host is a Syrian Baby Hamster Kidney (BHK) cell line.
50. The host of claim 49, wherein said cell line is available from ATCC under No. CRL 1632.
51. Recombinant human  $\alpha_2$ -macroglobulin of SEQ ID NO:2 or SEQ ID NO:4 in an active form.
52. Recombinant  $\alpha$ -macroglobulin, variants, fragments or derivatives thereof produced by a process of any of the claims 1 to 24.
53. Recombinant  $\alpha$ -macroglobulin, variants, fragments or derivatives thereof of claim 52 produced by the use of a vector of any of the claims 36 to 40.
54. Recombinant  $\alpha$ -macroglobulin, variants, fragments or derivatives thereof essentially as described.
55. Recombinant human  $\alpha_2$ -macroglobulin, variants, fragments or derivatives thereof essentially as described.
56. A growth medium comprising one or more  $\alpha$ -macroglobulins.

57. A growth medium comprising recombinant  $\alpha$ -macroglobulin, variants, fragments or derivatives thereof according to any of the claims 51 to 55.
58. Use of recombinant  $\alpha$ -macroglobulin, variants, fragments or derivatives thereof according to any of the claims 51 to 55 as a protein carrier in enzyme replacement therapy.
59. Use of recombinant  $\alpha$ -macroglobulin, variants, fragments or derivatives thereof according to any of the claims 51 to 55 as a DNA carrier in gene therapy.

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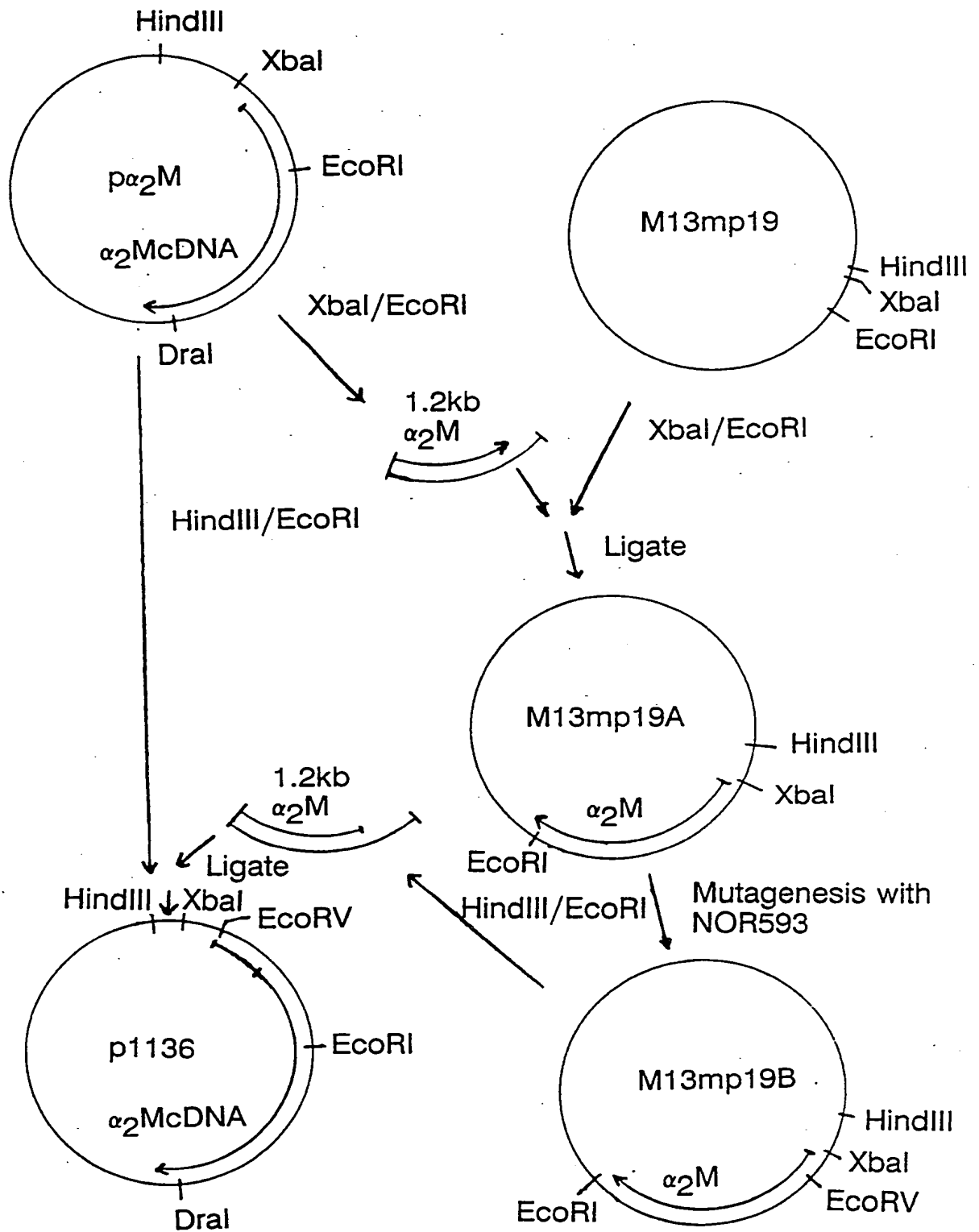


FIG. 1A

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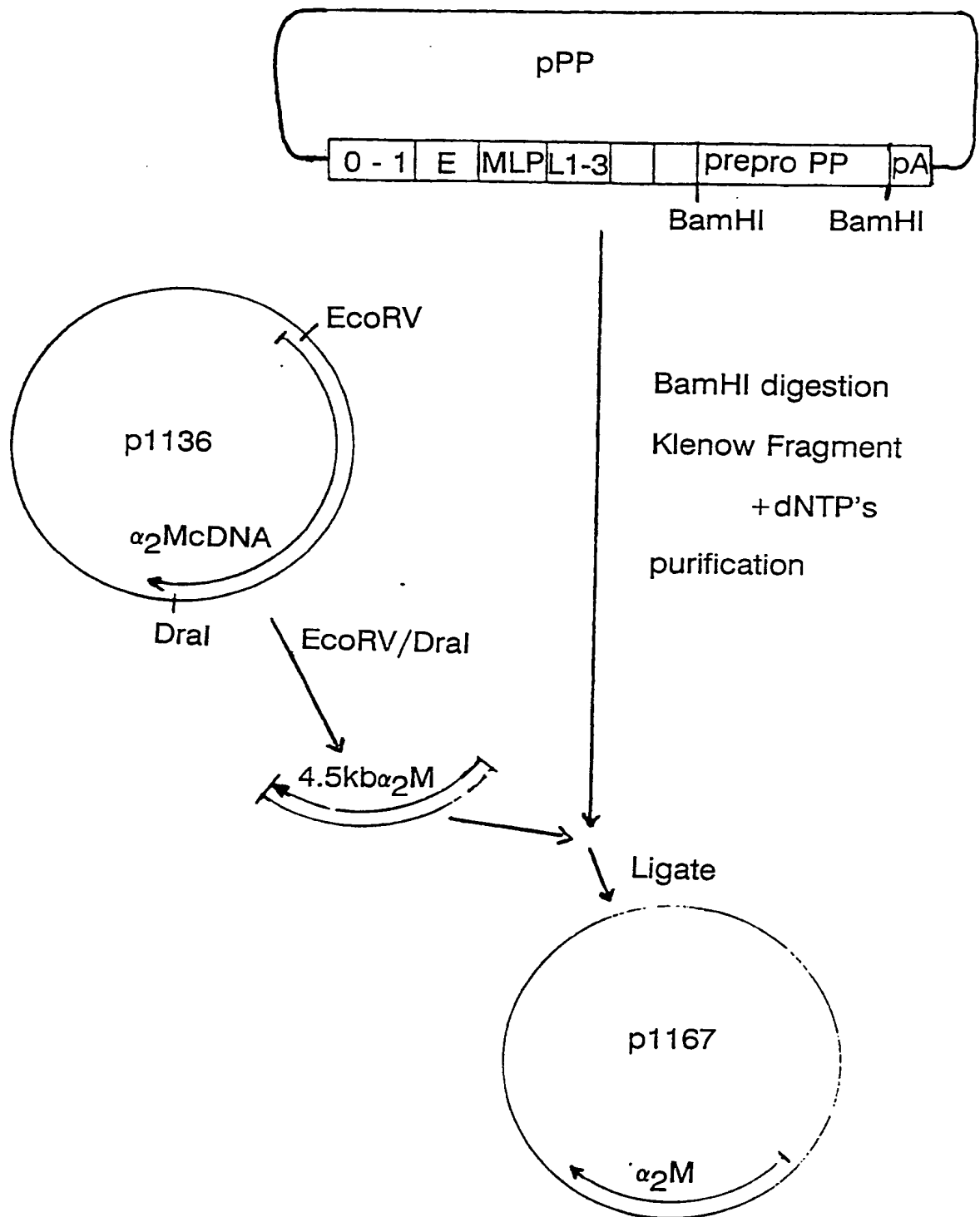


FIG. 1B

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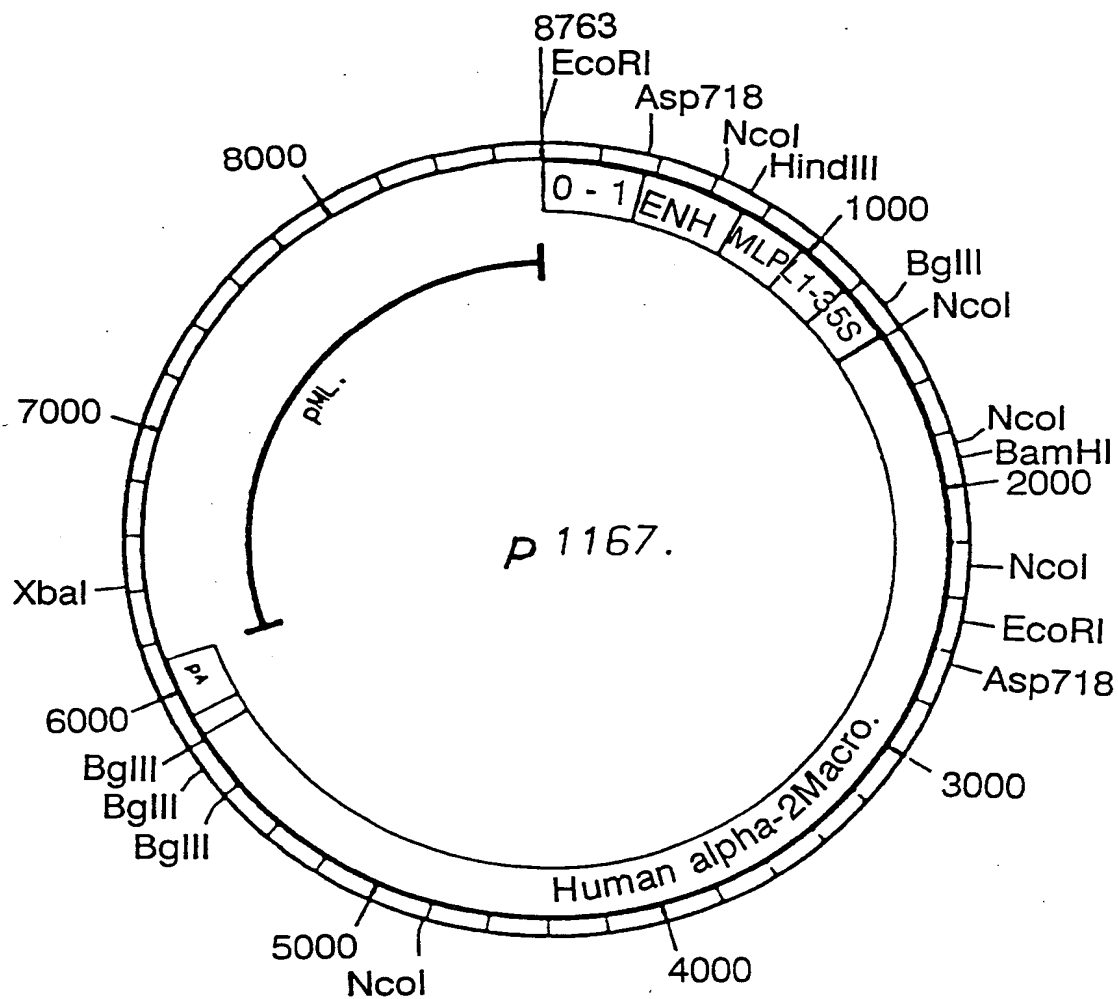


FIG. 2

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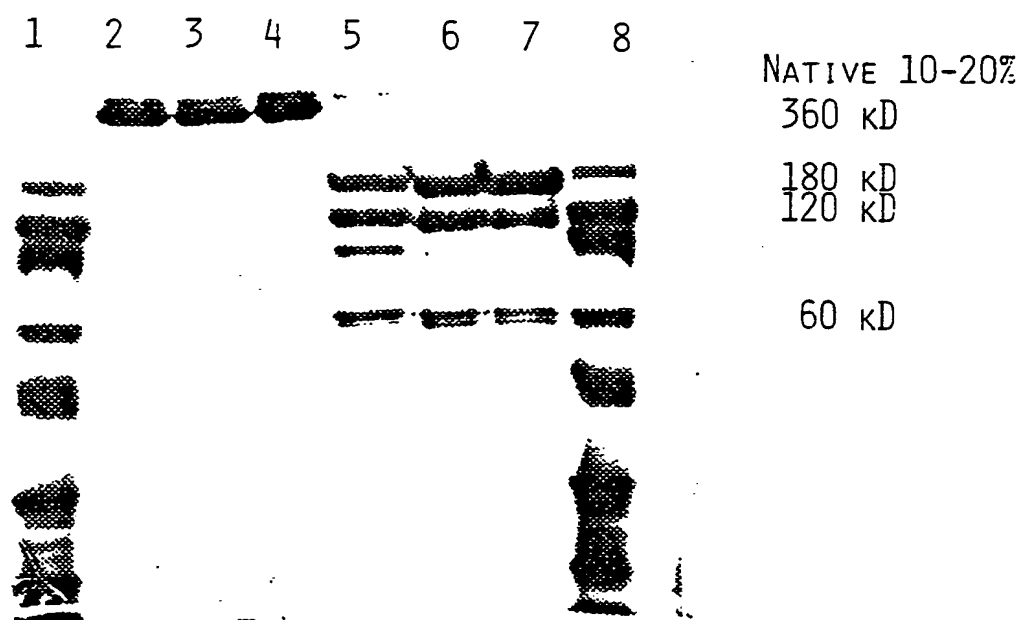


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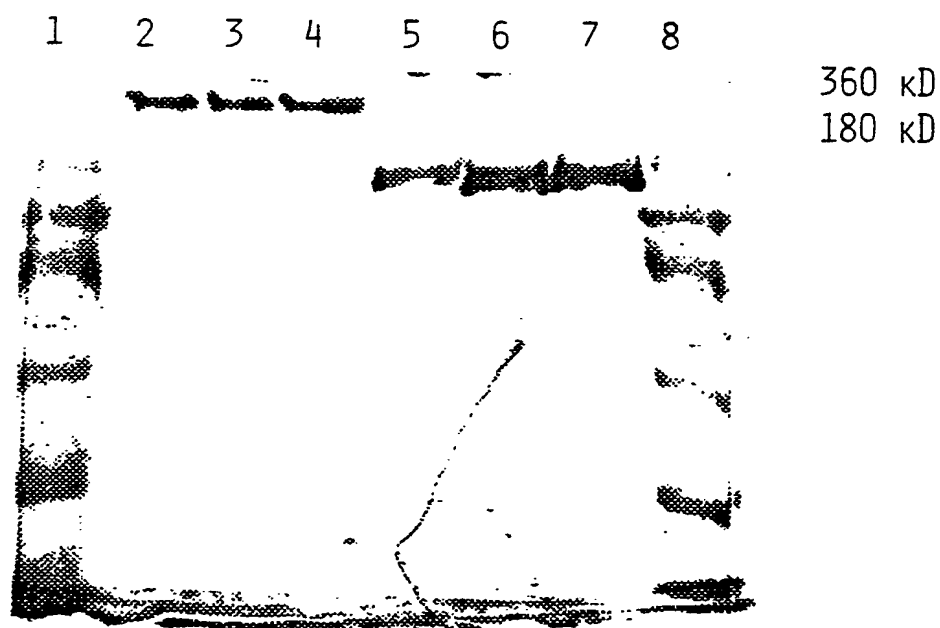


FIG. 4

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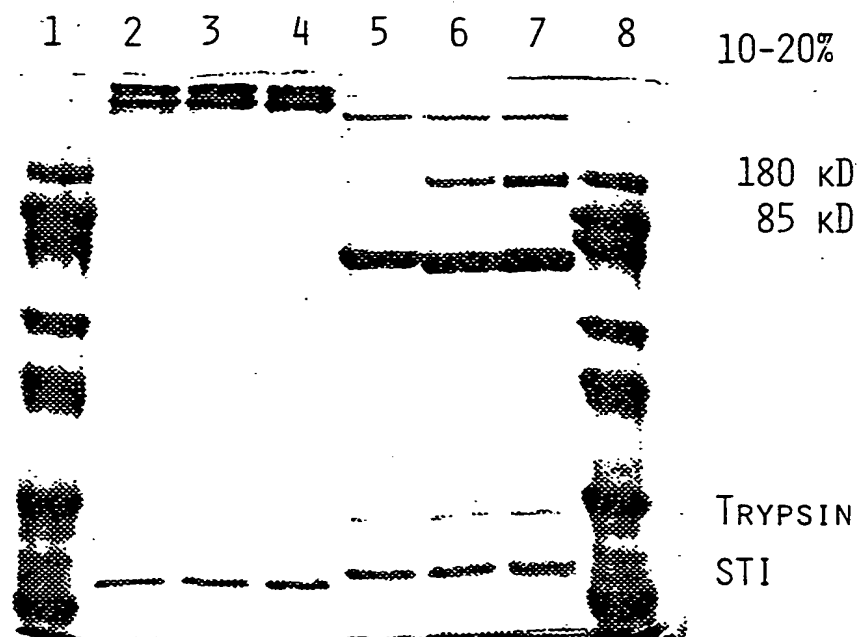


FIG. 5

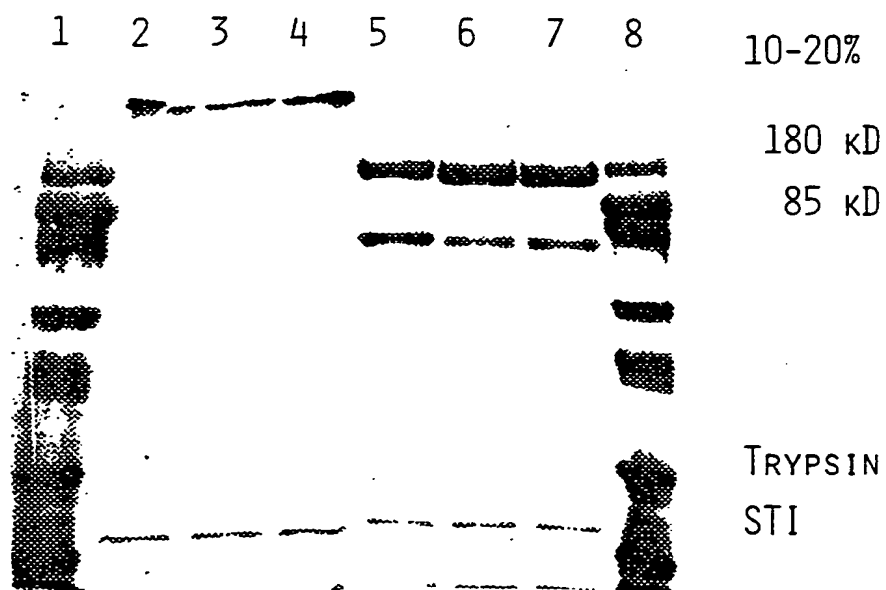
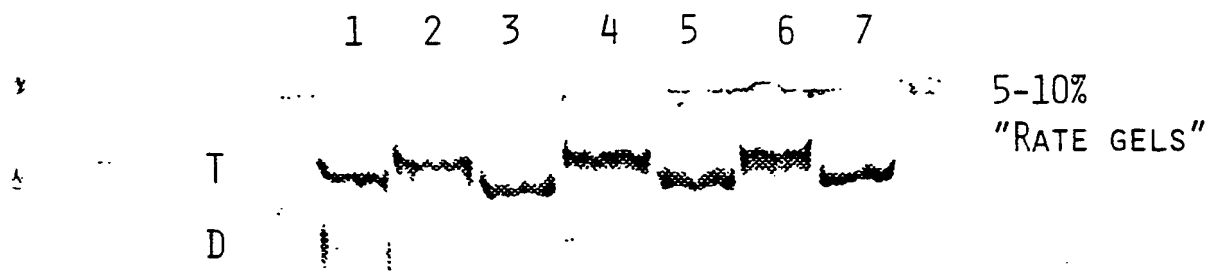


FIG. 6

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**FIG. 7**

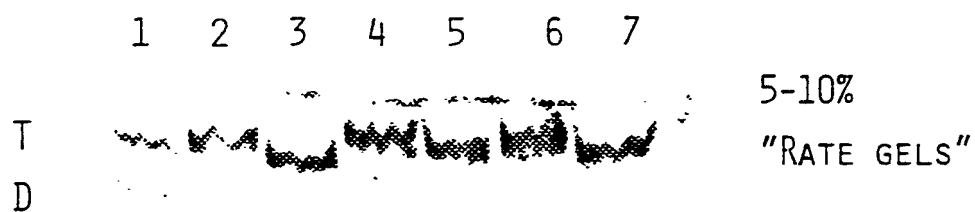


FIG. 8



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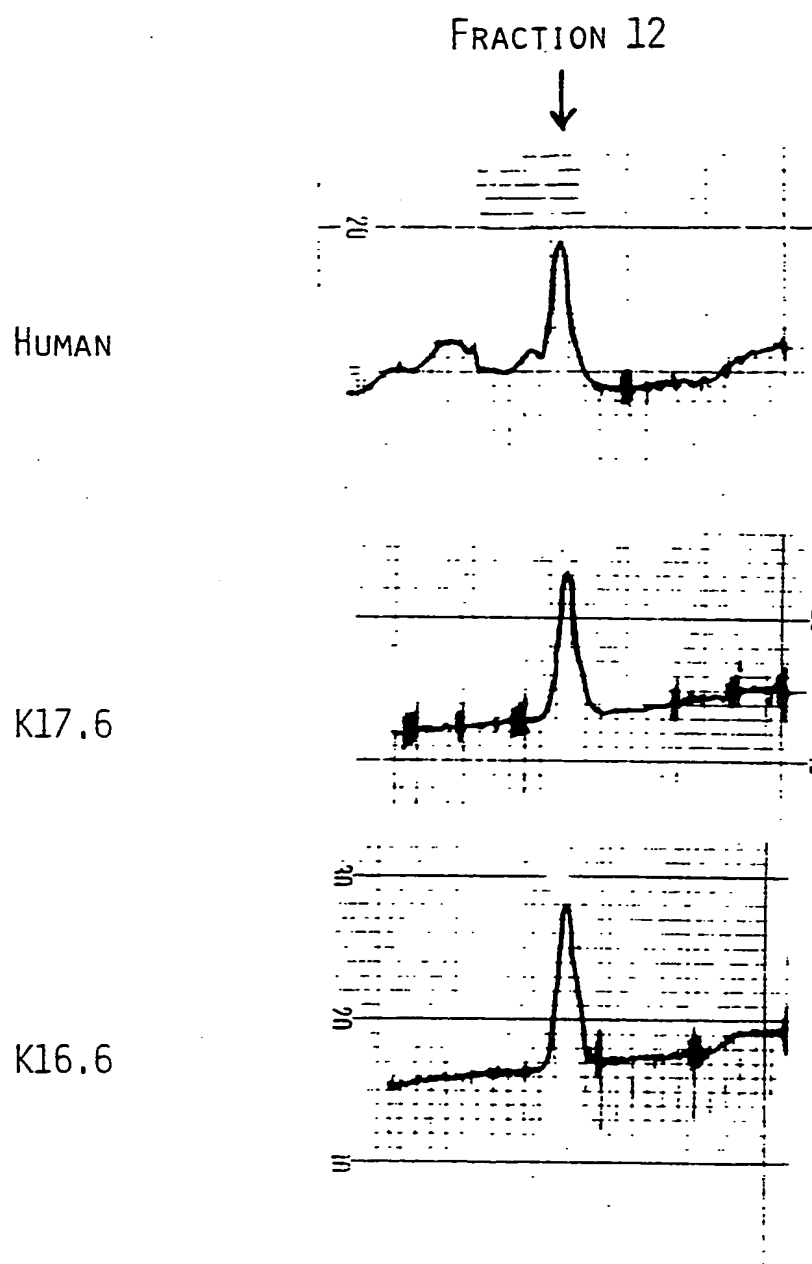


FIG. 9

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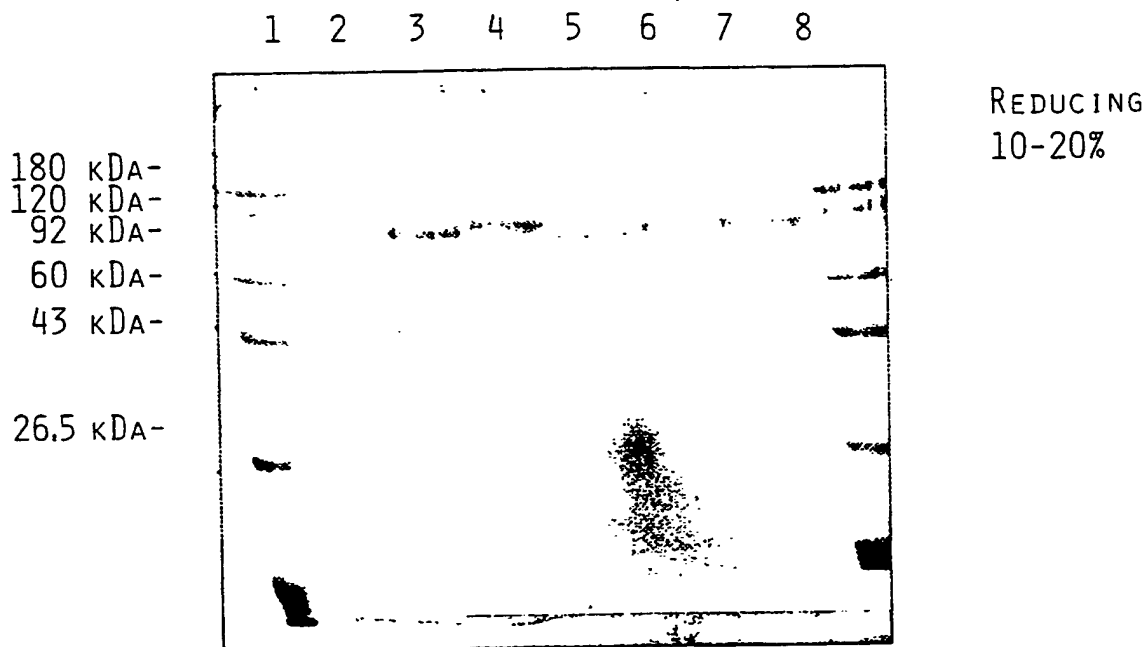


FIG. 10

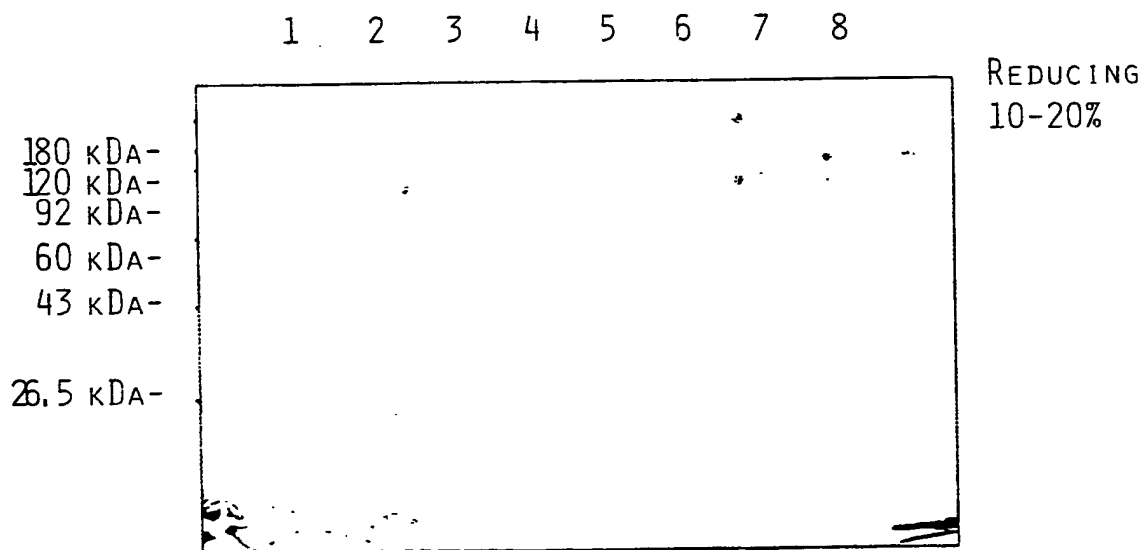


FIG. 11

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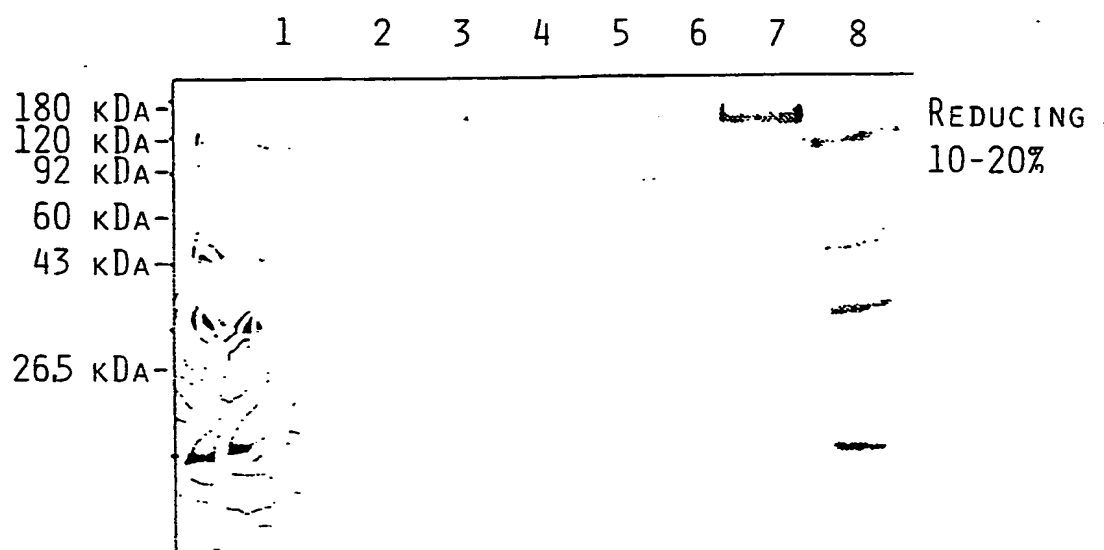


FIG. 12

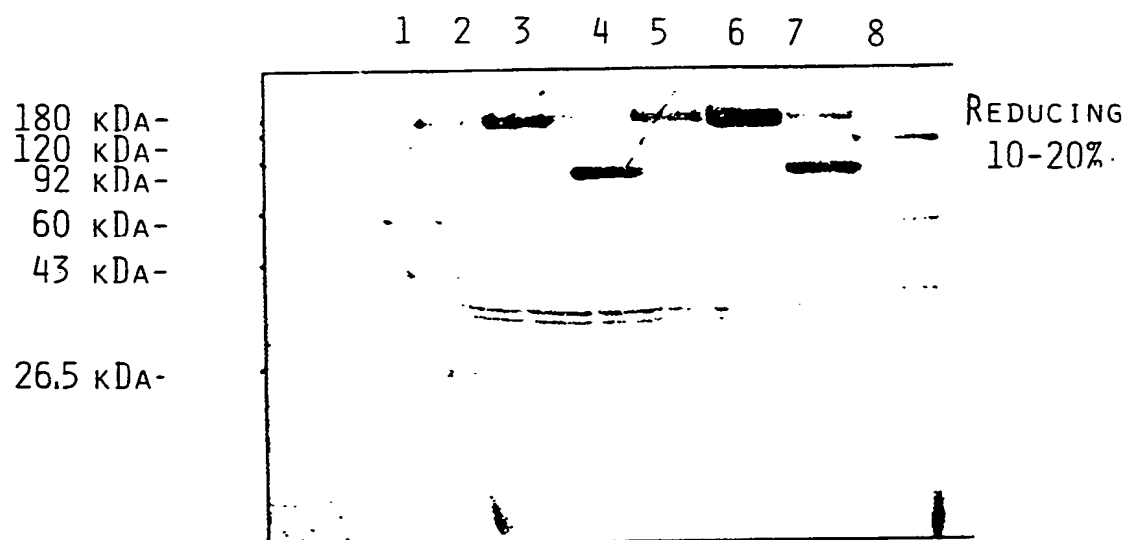


FIG. 13

# INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 90/00225

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup> According to International Patent Classification (IPC) or to both National Classification and IPC IPC5: C 12 N 15/15, A 61 K 37/64, C 07 K 13/00, C 12 P 21/02																	
<b>II. FIELDS SEARCHED</b> <div style="text-align: center; border: 1px solid black; padding: 2px;">Minimum Documentation Searched<sup>7</sup></div> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 25%; padding: 5px;">Classification System</th> <th style="padding: 5px;">Classification Symbols</th> </tr> <tr> <td style="padding: 5px; height: 40px; vertical-align: bottom;">IPC5</td> <td style="padding: 5px; vertical-align: bottom;">A 61 K; C 12 N; C 07 K</td> </tr> </table> <div style="text-align: center; border: 1px solid black; padding: 2px;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched<sup>8</sup></div> <p style="padding: 5px;">SE,DK,FI,NO classes as above</p>			Classification System	Classification Symbols	IPC5	A 61 K; C 12 N; C 07 K											
Classification System	Classification Symbols																
IPC5	A 61 K; C 12 N; C 07 K																
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b> <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%; padding: 5px;">Category *</th> <th style="width: 70%; padding: 5px;">Citation of Document,<sup>11</sup> with indication, where appropriate, of the relevant passages<sup>12</sup></th> <th style="width: 20%; padding: 5px;">Relevant to Claim No.<sup>13</sup></th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">X</td> <td style="padding: 5px;">Proc Natl Acad Sci USA, Vol. 82, No. 8, 1985 Kan, Chen Chen et al.: "Nucleotide sequence of cDNA encoding human alpha-2-macroglobulin and assignment of the chromosomal locus", see page 2282 - page 2286</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1,2,4, 14-28, 36-50, 52-59</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="text-align: center; vertical-align: top; padding: 5px;">--</td> <td style="text-align: center; vertical-align: top; padding: 5px;">5-10,29-34</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="padding: 5px;">Chemical Abstracts, volume 96, no. 15, 12 April 1982, (Columbus, Ohio, US), Mortensen, steen Bet al.: "Primary and secondary cleavage sites in the bait region of alpha-2-macroglobulin ", see page 253, abstract 117756z, &amp; FEBS Lett 1981, 135( 2), 295-300</td> <td style="text-align: center; vertical-align: top; padding: 5px;">5-10,29-34</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">A</td> <td style="padding: 5px;">Chemical Abstracts, volume 95, no. 7, 17 August 1981, (Columbus, Ohio, US), Sottrup-Jensen, Lars et al.: "Primary structure of the 'bait' region for proteinases in alpha-2-macroglobulin. Nature of the complex ", see page 261, abstract 57059s, &amp; FEBS Lett 1981, 127( 2), 167-173</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-59</td> </tr> </tbody> </table> <div style="display: flex; justify-content: space-between; font-size: 0.8em; padding: 5px;"> <div style="width: 45%;"> <p>* Special categories of cited documents:<sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>			Category *	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>	X	Proc Natl Acad Sci USA, Vol. 82, No. 8, 1985 Kan, Chen Chen et al.: "Nucleotide sequence of cDNA encoding human alpha-2-macroglobulin and assignment of the chromosomal locus", see page 2282 - page 2286	1,2,4, 14-28, 36-50, 52-59	Y	--	5-10,29-34	Y	Chemical Abstracts, volume 96, no. 15, 12 April 1982, (Columbus, Ohio, US), Mortensen, steen Bet al.: "Primary and secondary cleavage sites in the bait region of alpha-2-macroglobulin ", see page 253, abstract 117756z, & FEBS Lett 1981, 135( 2), 295-300	5-10,29-34	A	Chemical Abstracts, volume 95, no. 7, 17 August 1981, (Columbus, Ohio, US), Sottrup-Jensen, Lars et al.: "Primary structure of the 'bait' region for proteinases in alpha-2-macroglobulin. Nature of the complex ", see page 261, abstract 57059s, & FEBS Lett 1981, 127( 2), 167-173	1-59
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X	Proc Natl Acad Sci USA, Vol. 82, No. 8, 1985 Kan, Chen Chen et al.: "Nucleotide sequence of cDNA encoding human alpha-2-macroglobulin and assignment of the chromosomal locus", see page 2282 - page 2286	1,2,4, 14-28, 36-50, 52-59															
Y	--	5-10,29-34															
Y	Chemical Abstracts, volume 96, no. 15, 12 April 1982, (Columbus, Ohio, US), Mortensen, steen Bet al.: "Primary and secondary cleavage sites in the bait region of alpha-2-macroglobulin ", see page 253, abstract 117756z, & FEBS Lett 1981, 135( 2), 295-300	5-10,29-34															
A	Chemical Abstracts, volume 95, no. 7, 17 August 1981, (Columbus, Ohio, US), Sottrup-Jensen, Lars et al.: "Primary structure of the 'bait' region for proteinases in alpha-2-macroglobulin. Nature of the complex ", see page 261, abstract 57059s, & FEBS Lett 1981, 127( 2), 167-173	1-59															
<b>IV. CERTIFICATION</b> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; padding: 5px;">           Date of the Actual Completion of the International Search  <b>11th December 1990</b> </td> <td style="width: 50%; padding: 5px;">           Date of Mailing of this International Search Report  <b>1990 -12- 13</b> </td> </tr> <tr> <td style="width: 50%; padding: 5px; vertical-align: bottom;">           International Searching Authority  <div style="text-align: center; padding-top: 10px;"><b>SWEDISH PATENT OFFICE</b></div> </td> <td style="width: 50%; padding: 5px; vertical-align: bottom;">           Signature of Authorized Officer  <div style="text-align: center; padding-top: 10px;">   <b>Yvonne Siösteen</b> </div> </td> </tr> </table>			Date of the Actual Completion of the International Search <b>11th December 1990</b>	Date of Mailing of this International Search Report <b>1990 -12- 13</b>	International Searching Authority <div style="text-align: center; padding-top: 10px;"><b>SWEDISH PATENT OFFICE</b></div>	Signature of Authorized Officer <div style="text-align: center; padding-top: 10px;">   <b>Yvonne Siösteen</b> </div>											
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International Searching Authority <div style="text-align: center; padding-top: 10px;"><b>SWEDISH PATENT OFFICE</b></div>	Signature of Authorized Officer <div style="text-align: center; padding-top: 10px;">   <b>Yvonne Siösteen</b> </div>																

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Chemical Abstracts, volume 101, no. 11, 10 September 1984, (Columbus, Ohio, US), Sottrup-Jensen, Lars et al.: "Primary structure of human alpha-2-macroglobulin. V. The complete structure ", see page 237, abstract 85952p, & J. Biol. Chem. 1984, 259(13), 8318-8327 --	1-59
P	Chemical Abstracts, volume 111, no. 23, 4 December 1989, (Columbus, Ohio, US), Sottrup-Jensen, Larset al.: "The alpha-macroglobulin bait region. Sequence diversity and localization of cleavage sites for proteinases in five mammalian alpha-macroglobulins ", see page 227, abstract 210722y, & J. Biol. chem. 1989, 264(27), 15781-15789 --	5-10,29-34
P	Chemical Abstracts, volume 112, no. 25, 18 June 1990, (Columbus, Ohio, US), Marynen, P et al.: "A genetic polymorphism in a functional domain of human pregnancy zone protein: the bait region. Genomic structure of the bait domains of human pregnancy zone protein and alpha-2-macroglobulin ", see page 167, abstract 230679p, & FEBS Lett. 1990, 262( 2), 349-352 -- -----	5-10,29,34

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